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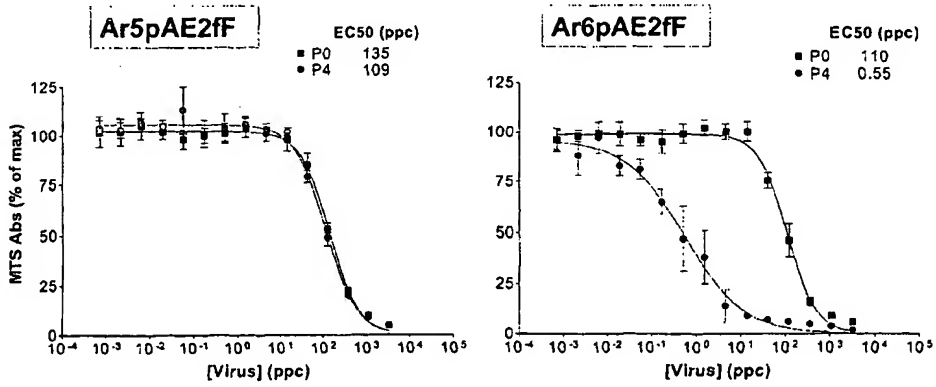
(54) Title: ASSAY TO DETECT REPLICATION COMPETENT VIRUSES




(57) Abstract: A biological assay for detecting a non-selective replication competent virus (RCV) in a preparation of selectively-replicating virus. The method comprises -passaging the virus preparation at least once on cells that differentially amplify the selectively-replicating virus versus a replication competent virus and further analyzing the virus preparation, thereby detecting the presence of a replication competent virus. The further analysis of the virus preparation includes one or more of the following: detecting increased cytopathic effect; detecting increased virus production; detecting increased potency to kill normal cells; detecting an altered restriction digest pattern; detecting an altered viral genome sequence; detecting anticipated recombinants with PCR amplification; and detecting acute hepatotoxicity in an animal administered the virus preparation. In one embodiment, the assay detects replication competent adenoviruses in a preparation of an oncolytic adenovirus.

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# ASSAY TO DETECT REPLICATION COMPETENT VIRUSES

## RELATED APPLICATIONS

Benefit of priority is claimed under 35 U.S.C. §119(e) to U.S. provisional application Serial No. 60/459,375, filed March 27, 2003, entitled "ASSAY TO DETECT REPLICATION COMPETENT ADENOVIRUSES," to Mark Bowe, Russette M. Lyons, and Tracey Walker, and to U.S. provisional application Serial No. 60/387,251, filed June 7, 2002 entitled "ASSAY TO DETECT REPLICATION COMPETENT ADENOVIRUSES," to Mark Bowe, Russette M. Lyons, and Tracey Walker. Both of these applications are incorporated herein by reference in their entireties.

## FIELD OF THE INVENTION

The present invention generally relates to methods and assays useful for detecting replication competent viruses in preparations of selectively replicating viruses.

## BACKGROUND OF THE INVENTION

Viruses that replicate selectively in tumor cells are being developed as anticancer agents ("oncolytic viruses"). Such oncolytic viruses amplify the input virus dose due to viral replication in the tumor, leading to spread of the virus throughout the tumor mass. In situ replication of viruses leads to cell lysis. This in situ replication may allow relatively low, non-toxic doses to be highly effective in the selective elimination of tumor cells.

These oncolytic viral viruses can be based on any virus that can be designed to preferentially replicate in tumors cells. Non-limiting examples of viruses designed to preferentially replicate in tumors cells include those derived from adenoviruses (US patent 5,998,205; WO 02/067861), herpesvirus (PCT Publication number WO 96/39841), reovirus (Yin, H.S.,. J Virol Methods, 1997. 67:93- 101; Strong, J.E. and P.W. Lee,. J Virol, 1996. 70:612-616; Strong, J.E., et al, Virology, 1993. 197:405-411; Minuk, G.Y., et al., J Hepatol, 1987. 5:8-13; Rozee, K.R., et al., Appl Environ Microbiol, 1978. 35:297-300; Coffey, M.C., et al., Science, 1998. 282:1332-1334; Strong, J.E., et al., Embo J, 1998 17:3351-1362; Mundschau, L.J. and D.V. Faller, J Biol Chem, 1992. 267:23092-23098), Paramyxovirus (e.g., Newcastle Disease,

measles and mumps virus; PCT Publication number WO 99/18799), Togavirus (e.g., Sindbis PCT Publication number WO 99/18799), Parvoviruses, Poxvirus (e.g., Vaccinia), Picornavirus (e.g., Poliovirus), Orthonyxoviruses (e.g., influenza), and rhabdovirus (PCT Publication number WO 01/19380).

One approach to achieving selectivity is to introduce loss-of-function mutations in viral genes that are essential for growth in non-target cells but not in tumor cells. This strategy is exemplified by the use of Addl1520, which has a deletion in the E1b-55KD gene. (WO 94/189992; Ganly, et al., "A Phase I Study of Onyx-015, an E1B Attenuated Adenovirus, Administered Intratumorally to Patients with Recurrent Head and Neck Cancer," Clinical Cancer Research, 6:798-806 (March 2000). In normal cells, the adenoviral E1b-55KD protein is needed to bind to p53 to prevent apoptosis. In p53-deficient tumor cells, E1b-55K binding to p53 is unnecessary. Thus, deletion of E1b-55KD should theoretically restrict virus replication to p53-deficient tumor cells.

Another approach is to use tissue-selective promoters to control the expression of one or more early viral genes required for replication (WO 96/17053; WO 99/25860; WO 02/067861; and WO 02/068627). Thus, in this approach the adenoviruses will selectively replicate and lyse tumor cells if a gene that is essential for replication is under the control of a promoter or other transcriptional regulatory element that is tissue-selective.

One concern when using an adenoviral virus preparation is the possibility of contamination with a replication competent adenovirus (RCA) or even a non adenoviral replication competent virus. When the adenovirus is a standard replication defective virus (e.g., ΔE1, ΔE2, and/or ΔE4), detection of RCA can usually be accomplished using either PCR methods or biological cell-based assays (Dion, et al., "Supernatant rescue assay vs. polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks," *J. Virol Methods*, 56(1):99-107 (January 1996); Murakami, et al., "A single short stretch of homology between adenoviral vector and packaging cell line can give rise to cytopathic effect-inducing, helper-dependent e1-positive particles," *Hum Gene Ther*, 13(8):909-920 (May 20, 2002)). The PCR methods utilize primers based on predicted recombination events that could lead to the production of RCA; for example, recombination events with the genome of the production cell line, wherein the complementing gene

provided by the cell line is recombined into the adenovirus, thus creating an RCA. These PCR-based assays are limited to only detecting predicted recombination events. The cell-based assays, when used with a replication defective virus, comprise infecting non-complementing cell lines with the virus preparations and serially passaging the virus on the non-complementing cell line (Dion, et al., *supra*). The desired replication defective virus should not replicate on the non-complementing cell line, whereas an RCA would amplify to a detectable level through serial passaging. Detection of the RCA can usually be determined through the appearance of cytopathic effect of the cells during the amplification steps. This biological cell-based assay should theoretically detect the presence of any recombinant or contaminant RCAs, whereas the PCR-based assays only detect predicted RCAs. The present invention is meant to include any selectively replicating virus and not just "oncolytic viruses." This would include any virus that preferentially replicates in a specific tissue or cell type.

It is therefore an object of the present invention to provide a method for detecting replication competent viruses in preparations of selectively replicating viruses.

## SUMMARY OF THE INVENTION

The present invention addresses the need for a biological assay to detect replication competent virus (RCV) in replication selective virus (a.k.a. selectively replicating; e.g., oncolytic virus) preparations.

Selectively replicating viruses present a unique challenge with regard to testing for the presence of RCV. Unlike replication defective viruses, selectively replicating adenoviruses are replication competent. The degree of replication of selectively replicating viruses will depend on both the design of the virus and the cells infected. It is unlikely that cell lines can be identified that will support efficient replication of a fully replication competent virus while being absolutely non-permissive for replication of a selectively replicating virus such that testing similar to that currently used for replication defective viruses can be applied to these novel products. Rather, a biological testing strategy based on the relative difference in replication between a fully replication competent virus and the selectively replicating virus is presented herein.

Accordingly, the present invention provides a method for detecting a non-selective replication competent virus (RCV) in a preparation of selectively-replicating virus, comprising:

(a) passaging the virus preparation at least once on cells that differentially amplify the selectively-replicating virus versus a replication competent virus; and

(b) analyzing the virus preparation amplified in (a), thereby detecting the presence of a replication competent virus, wherein analyzing the virus preparation comprises one or more of the following: detecting increased cytopathic effect; detecting increased virus production; detecting increased potency to kill normal cells; detecting an altered restriction digest pattern; detecting an altered viral genome sequence; detecting anticipated recombinants with PCR amplification; and detecting acute hepatotoxicity in an animal administered the virus preparation.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic of PCR detection promoter recombinations.

Figure 2 shows the potency of Ar6pAE2fE3F as compared to wild-type Ad5 on HS-68 cells. The difference in potency (LD50 values) is approximately 30 fold.

Figure 3 shows the potency of Ar6pAE2fE3F (P0), Ar6pAE2fE3F (P4) and Ad5 wild-type on MRC-5 cells. Potency of virus is measured by cell killing with an MTS readout. Potency increased more than 2 logs after amplification on MRC-5 cells. Potency is greater than wild-type Ad5 on MRC-5 cells.

Figure 4 shows the potency on MRC-5 cells of Ad5 wild-type after passaging on MRC-5 cells. No significant change in Ad5 potency after amplification was observed.

Figure 5 shows the structure/sequence some of the RCAs generated in the assay from Ar6pAE2fE3F. Right end of rearranged vector contains the packaging signal, suggesting recombination mechanisms of either intermolecular recombination or polymerase jumping. These RCAs had a deletion of some or all of E2F promoter, deletion of the p(A), duplication of part or all of E4 promoter and/or duplication of the packaging signal.

Figure 6 shows a comparison of cell killing (potency) by MTS.

Figure 7 shows a comparison of replication measured by hexon FACS assay in human cells.

Figure 8 shows the potency on MRC-5 cells for Ar5pAE2fF and Ar6pAE2fF.

Figure 9 shows potency on MRC-5 and human aortic endothelial cells for Ar6pAE2fE3F and wild-type Ad5 virus.

Figure 10 shows schematic representations of selectively replicating, oncolytic adenoviral vectors Ar6pAE2fE3F, Ar6pAE2fF and Ar5pAE2fF. The Ar6pAE2fE3F vector is based on the human adenovirus serotype 5 (Ad5) backbone, and is approximately 34kb in size (Jakubczak et al. 2002). The native adenoviral E1A promoter has been replaced with the human E2F-1 promoter (E2f P). The packaging signal (ψ) has been moved to the right end of the genome, and an SV40 polyadenylation sequence (pA) has been inserted after the left inverted terminal repeat (ITR). The 14.7K gene of the E3 region has also been deleted. The Ar6pAE2fF vector is similar to Ar6pAE2fE3F, except that the entire adenoviral E3 region (E3) has been removed. The Ar5pAE2fF vector is similar to Ar6pAE2fF, except that the packaging signal remains at the left end of the genome. Schematics are not drawn to scale. E1A, adenoviral E1A coding sequence; E4, adenoviral E4 coding sequence.

Figure 11 shows a potency analysis by cytotoxicity assay. Wild-type Ad5 and Ar6pAE2fE3F, either before (P0) or after (P4) four passages on MRC-5 cells, are diluted to the concentrations shown (ppc; particles per cell) and applied to MRC-5 cells in 96-well plates. The cells are incubated for 10 or 7 days, respectively, and then analyzed for viability using an MTS assay. Measurements of viability at each dilution are expressed as the mean ± SEM (N = 4). EC50's are calculated by regression analysis of a four parameter logistic equation, using the pooled data for each vector.

Figure 12 shows a potency analysis by cytotoxicity assay. Wild-type Ad5 and Ar6pAE2fE3F, either before (P0) or after (P4) four passages on MRC-5 cells, are diluted to the concentrations shown (ppc; particles per cell) and applied to primary human aortic endothelial cells in 96-well plates. The cells are incubated for 10 or 7 days, respectively, and then analyzed for viability using an MTS assay. Measurements of viability at each dilution are expressed as the mean ± SEM (N = 4). EC50's are calculated by regression analysis of a four parameter logistic equation, using the pooled data for each vector.

Figure 13 shows the structure of Ar6pAE2fE3F recombinants. Twenty-six clonal isolates are prepared from the Ar6pAE2fE3F that has been passaged 4 times on MRC-5. Each clone is sequenced. Seven individual recombinants were found (A-G). The labeled schematic indicates the potential components of the recombined left end,

while the bars below indicate the sequence of each recombinant analyzed. The columns to the right indicate whether each recombinant has been isolated using PER.C6 or MRC-5 cells or both. The forward (→) and reverse (←) arrows indicate positions of the forward and reverse primers, respectively, for a quantitative PCR assay that recognizes recombinants A and E (REC133 Q-PCR), while the asterisked bar (*–) indicates the position of the probe. The schematics are not drawn to scale. ITR, inverted terminal repeat; pA, poly A site; E2f P, human E2F-1 promoter; E1A, adenoviral E1A coding sequence; E4, adenoviral E4 coding sequence; E4 P, adenoviral E4 promoter; Ψ packaging signal; Rec P, recombinant promoter.

Figure 14 shows a potency analysis by cytotoxicity assay. The vector indicated (Ar6pAE2fF or Ar5pAE2fF) is initially cloned and produced on the cell line indicated in parentheses (defined as P0), prior to being passaged sequentially four times on MRC-5 cells (P4). The vector samples are diluted to the concentrations shown (ppc; particles per cell) and applied to MRC-5 cells in 96-well plates. The cells are incubated for 10 days, and then analyzed for viability using an MTS assay. Measurements of viability at each dilution are expressed as the mean ± SE of four replicates. EC50's are calculated by regression analysis of a four parameter logistic equation, using the pooled data for each vector.

## DETAILED DESCRIPTION OF THE INVENTION

Just as for any viral vector or other therapeutic, safety testing is a component of both early development of replication-selective viruses and to release of individual lots for clinical use. Viruses have a propensity to recombine, and so a key concern with viral vectors is the potential presence of recombinant replication competent virus {Lochmuller, Jani, et al. 1994}{Hehir, Armentano, et al. 1996}{Smith & Eck 1999}{Murakami, Pungor, et al.}. Because a typical replication defective virus is deleted for one or more crucial genes, PCR may be used to test for the presence of recombinants containing the deleted gene {Zhang, Koch, et al. 1995}{Dion, Fang, et al. 1996}{Melcher, Murphy, et al. 1999}. A more general approach, and an advisable one due to its ability to detect the unexpected recombinant, is to use a biological assay to detect the replication competent recombinant {Dion, Fang, et al. 1996}{Hehir, Armentano, et al. 1996}{Zhu, Grace, et al. 1999}{Melcher, Murphy, et al. 1999}{Roitsch, Achstetter, et al.}{Murakami, Pungor, et al.}. A biological assay for

replication competent virus in a replication defective virus preparation typically involves inoculating a sufficient quantity of the virus into one or more cell lines which are non-permissive to the replication defective virus but permissive to the parental wild-type virus or replication competent recombinant. After passaging to amplify any replication competent virus that might be present, the cultures are examined for evidence of viral infection. Cytopathic effect (CPE) is a common read-out for viral vectors in this type of assay {Lehmberg, McCaman, et al. 2002}. These assays are endpoint assays with established limits of detection that have been determined by spike-in experiments using known quantities of replication competent positive control virus.

Selectively replicating viruses present a unique challenge with regard to testing for the presence of replication competent virus. Unlike replication defective viruses, replication-selective viruses are to some degree replication competent. For example, the the tumor selective adenovirus Ar6pAE2fE3F, which is described in detail in PCT/US02/05280 (WO 02/068627) and PCT/US02/05300 (WO 02/067861), is a genetically modified human adenovirus designed to preferentially replicate in tumor cells, resulting in tumor cell death due to oncolysis. Ar6pAE2fE3F utilizes the E2F-1 promoter to control the expression of E1a. Although the E2F-1 promoter is permanently de-repressed in Rb-defective tumor cells, it is transiently de-repressed in normal, proliferating cells. Therefore, a low level of replication of replication-selective viruses controlled by the E2F-1 promoter may be expected in normal cells in vitro.

The E2F-1 promoter takes advantage of multiple defects in the Rb pathway, a common feature of many tumor cells, that results in hyper-phosphorylated Rb, release of Rb from E2F/DP-1 complexes and concomitant activation of promoters containing E2F binding domains, such as the E2F-1 promoter itself. Although the E2F-1 promoter is activated in Rb-defective tumor cells due to mutation or loss of RB pathway checkpoint proteins, RB is also transiently active during the cell cycle of normal cells. Therefore, a low level of replication of Ar6pAE2fE3F may be expected in normal cells in vitro.

In normal cells the RB pathway is tightly regulated. Without being bound by theory, the inventors believe that the mechanism of action is as follows. In G0 and G1, the ternary complex of E2F-DP-1-pRB represses transcription from promoters containing E2F binding sites, including the E2f-1 promoter in Ar6pAE2fE3F. pRB is

temporally regulated by phosphorylation during the cell cycle. Phosphorylation reversibly inactivates pRB, resulting in transcriptional activation by E2F-DP-1 dimers and entry into S phase of the cell cycle. Dephosphorylation of pRB after mitosis causes re-entry into G1 phase. In tumor cells, any one or several of the cell cycle checkpoint proteins may be modified and lead to cell cycle deregulation and unrestricted cell cycling. Loss of the pRB-E2F-DP-1 interaction, or abundance of "free E2F," results in derepression/activation of promoters having E2F sites. Derepression of the E2f-1 promoter in Ar6pAE2fE3F leads to transcription of E1a, viral replication, and oncolysis.

As shown in the examples below, the RCVs that can be generated can be unpredictable. The characteristics of each RCV and how they were generated will vary based on the specific design of the particular selctively-replicating virus. It is the entire viral genome that must be considered, as each element, whether engineered into the virus or an endogenous viral sequence, may influence the nature of any recombinant that is formed. These data in the provided examples suggest that a combination of factors contribute to the generation of RCV in a background of replication-selective virus. The relative importance of each of these factors may vary with each virus construct. An interpretation concerning virus structure to be made from the data in the examples is that recombination events can be surprising, thus calling for the use of sequence-independent biological assays, as described herein, for more comprehensive detection of undesirable recombinants that have formed.

The degree of replication of replication-selective viruses will depend on both the design of the virus and the cells that are infected. It is unlikely that cell lines can be identified that will support efficient replication of a non-selective replication competent virus while being absolutely non-permissive for replication of a selectively replicating viral vector. Therefore, biological assays that rely solely on the presence of, for example, CPE would be unlikely to be able to differentiate easily and objectively between a replication-selective vector and a replication competent virus.

Accordingly, in one aspect, the present invention provides a method for detecting a non-selective replication competent virus in a viral preparation. The method includes passaging the viral preparation at least once on cells. The cells can be any cells that are selected prior to performing the assay and they are selected for their ability to differentially amplify the selectively-replicating virus versus an RCV.

RCV may be defined as a recombinant or contaminant virus which lacks the selectivity of the replication-selective virus.

In one embodiment, the viral preparation is comprised of a virus from the group consisting of adenovirus, herpesvirus, reovirus, paramyxovirus, sindbis, parvoviruses, poxvirus, picornavirus, orthonyxoviruses, and rhabdovirus.

In a one embodiment, the viral preparation is an adenoviral preparation. When an adenoviral preparation is employed, detected RCV would most likely be a replication competent adenovirus (RCA), although the assay will detect a non-adenovirus RCV.

In another embodiment, the virus is a tissue-specific replication-conditional virus.

In another embodiment of the invention, the replication-conditional virus comprises a heterologous tissue-specific transcriptional regulatory sequence operatively linked to the coding region of a gene that is essential for replication of the virus, wherein the transcriptional regulatory sequence functions in the cell so that replication of the virus occurs in the cell.

In another embodiment of the invention the selectively-replicating virus comprises a heterologous tissue-specific transcriptional regulatory sequence operatively linked to the coding region of a gene that is essential for replication of the virus.

In another embodiment of the invention, the transcriptional regulatory sequence is selected from the group consisting of an E2F-responsive promoter, a human telomerase reverse transcriptase (hTERT) promoter, an osteocalcin promoter, a carcinoembryonic antigen (CEA) promoter, a DF3 promoter, an α-fetoprotein promoter, an ErbB2 promoter, a surfactant promoter, a tyrosinase promoter, a MUC1/DF3 promoter, a TK promoter, a p21 promoter, a cyclin promoter, an HKLK2 promoter, a uPA promoter, a HER-2neu promoter, a prostate specific antigen (PSA) promoter, a probasin promoter, a glandular kallikrein transcriptional regulatory element (U.S. Patent Application Publication No. US 2002/0136707 A1), a uroplakin II-derived transcriptional regulatory element (U.S. Patent Application Publication No. US 2002/0120117 A1), and a melanoma cell specific transcriptional regulatory element (TRE), e.g. a tyrosinase, tyrosinase-related protein 2 (TRP2), MIA, microphthalmia associated transcription factor (MITF); melanocyte-specific gene 1;

melanocyte-specific tyrosinase-related protein-1, or MART-1 derived TRE (all disclosed in U.S. Patent Application Publication No. US 2003/0039633 A1).

In another embodiment of the invention, the transcriptional regulatory sequence is an E2F-1 promoter.

In another embodiment of the invention, the virus further comprises a second heterologous tissue-specific transcriptional regulatory sequence operatively linked to the coding region of a second gene that is essential for replication of the virus, wherein the second transcriptional regulatory sequence functions in the cell so that replication of the virus occurs in the cell. In various embodiments, the first and second heterologous tissue-specific transcriptional regulatory sequences are the same or different.

In another embodiment of the invention, the selectively-replicating virus comprises first and second genes co-transcribed as a single mRNA, wherein the first and the second genes are under transcriptional control of a heterologous, target cell-specific TRE, wherein the second gene has a mutation in or deletion of its endogenous promoter and is under translational control of an internal ribosome entry site (IRES) (WO 01/73093).

In another embodiment of the invention, the virus further comprises a heterologous coding sequence. In one embodiment, the product of the coding sequence provides anti-tumor activity in the cells of a target tissue. In one embodiment, the heterologous coding sequence encodes a GM-CSF.

In another embodiment of the invention, the virus is replication-competent in a neoplastic cell and overexpresses an adenovirus death protein.

In another embodiment of the invention, the tissue-specific replication-conditional virus is an adenovirus.

In another embodiment of the invention, the tissue-specific replication-conditional adenovirus is tumor-specific.

In a preferred embodiment the tissue-specific replication-conditional virus or the tumor-specific replication-conditional virus is an adenovirus.

In another embodiment of the invention, the tumor-specific replication-conditional adenovirus comprises a mutation or deletion in the E1b gene, wherein the encoded E1b protein lacks the capacity to bind p53.

In another embodiment of the invention, the tumor-specfic replication-conditional adenovirus comprises a mutation or deletion in the E1b gene. Preferably,

the encoded E1b protein lacks the capacity to bind p53. For examples, See U.S. Pat. No. 5,677,178. This modification of the E1b region may be combined with viruses where all or a part of the E3 region is present.

In another embodiment of the invention, the tumor-specific replication-conditional adenovirus comprises a mutation or deletion in the E1a gene. For examples, see U.S. Pat. No. 5,677,178. Preferably the encoded E1a protein lacks the capacity to bind RB. This modification of the E1a region may be combined with viruses where all or a part of the E3 region is present.

In another embodiment of the invention, the selectively-replicating virus comprises (a) an E3 sequence; and (b) an E1A gene under transcriptional control of a probasin transcriptional regulatory element and an E1B gene under transcriptional control of a prostate specific antigen transcriptional regulatory element. (WO 00/39319). For example, the adenovirus vector comprises the entire E3 region, the probasin transcriptional regulatory element is the rat probasin promoter, and the prostate specific antigen transcriptional regulatory element is the human prostate-specific enhancer/promoter. (Yu et al., Cancer Research 59:4200-4203, 1999).

In another embodiment, the replication-conditional adenovirus comprises a mutation or deletion in the E3 region. However, in an alternative embodiment, all or a part of the E3 region may be preserved or re-inserted. See, e.g., U.S. Pat. No. 6,495,130. Presence of all or a part of the E3 region may decrease the immunogenicity of the virus. It also may increase cytopathic effect in tumor cells and decrease toxicity to normal cells. Preferably, the virus expresses more than half of the E3 proteins.

In one embodiment of the invention, the transcriptional regulatory sequence is a promoter or an enhancer.

In another embodiment of the invention, the adenovirus coding region that is operatively linked to the transcriptional regulatory sequence is an E1, E2, or E4 coding region. In various embodiments, the E1 coding region is an E1a or E1b coding region. In other various embodiments, the E2 coding region is an E2a or E2b coding region.

In another embodiment of the invention, the adenovirus comprises an E2F-1 promoter operatively linked to the E1a coding region.

In another embodiment of the invention, the adenovirus comprises an E2F-1 promoter operatively linked to the E1a coding region and an hTERT promoter operatively linked to the E4 coding region.

In another embodiment of the invention, the adenovirus comprises an adenovirus early gene essential for propagation under the transcriptional control of a prostate cell specific response element for transcription of prostate specific antigen comprising an enhancer and promoter specific for a prostate cell.

In another embodiment of the invention, the adenovirus comprises at least one of the genes E1A, E1B, or E4 under the transcriptional control of a prostate cell specific response element

In another embodiment of the invention, the adenovirus comprises a transgene under the transcriptional control of a prostate cell specific response element and lacking at least one of E1A, E1B, or E4 as a functional gene.

In another embodiment of the invention, the adenovirus is replication competent only in mammalian cells expressing prostate specific antigen.

In another embodiment of the invention, the adenovirus comprises an adenovirus gene essential for propagation under transcriptional control of a prostate specific response element, said prostate cell specific response element comprising an enhancer specific for prostate specific antigen and a promoter.

In another embodiment of the invention, the adenovirus comprises E1A and E1B, wherein E1A and E1B arc both under transcriptional control of separate a fetoprotein transcription regulatory elements .

In another embodiment of the invention, the adenovirus comprises a first adenovirus gene under transcriptional control of a first heterologous transcriptional regulatory element (TRE) and at least a second gene under transcriptional control of a second heterologous TRE, wherein the first heterologous TREs is cell-specific, the first heterologous TRE is different from the second heterologous TRE, and the heterologous TREs are functional in the same cell.

In another embodiment of the invention, the adenovirus comprises an adenovirus gene under transcriptional control of an alpha-fetoprotein transcription regulatory element .

In another embodiment of the invention, the adenovirus comprises an adenovirus gene under transcriptional control of a transcriptional regulatory element comprising a cell status- specific TRE.

In another embodiment of the invention, the adenovirus comprises (a) an adenovirus gene under transcriptional control of a target cell- specific transcriptional regulatory element; and (b) an E3 sequence.

In another embodiment of the invention, the adenovirus comprises g (a) an E3 sequence; and (b) a first adenovirus gene under transcriptional control of a first target cell-specific transcriptional response element and a second gene under transcriptional control of a second target cell-specific TRE.

In another embodiment of the invention, the adenovirus comprises a therapeutic gene and a disease specific gene regulatory region operationally linked to at least one replication gene wherein the cancer cells activate the tumor specific gene regulatory region causing the adenoviral vector to replicate.

In another embodiment of the invention, the adenovirus possesses enhanced infectivity towards a specific cell type due to a modification or replacement of the fiber of a wildtype adenovirus, said modification or replacement resulting in enhanced infectivity relative to said wildtype adenovirus, and wherein said infectivity-enhanced conditionally-replicative adenovirus has at least one conditionally regulated early gene, said early gene conditionally regulated such that replication of said infectivity-enhanced conditionally-replicative adenovirus is limited to said specific cell type.

In another embodiment of the invention, the adenovirus is derived from a human adenovirus 5 genome or a human adenovirus 35 genome.

In describing the present invention, the following terms are employed and are intended to be defined as indicated below.

As used herein, the terms "virus" and "viral particle" are used to include any and all viruses that infect a human or anima cells.

As used herein, the terms "virus preparation" and "viral preparation" are used interchangeably and refer to any batch of a virus. This includes batches prepared for research, preclinical or clinical use. A viral preparation may be purified, concentrated, non-purified or partially purified.

As used herein, the terms "adenovirus" and "adenoviral particle" are used to include any and all viruses that may be categorized as an adenovirus, including any adenovirus that infects a human or an animal, including all groups, subgroups, and serotypes. Adenovirus serotypes 1 through 47 are currently available from ATCC and the invention contemplates the production of any other serotype of adenovirus available from any source. The adenoviruses that can be produced according to the

invention may be of human or non-human origin. For instance, an adenovirus can be of subgroup A (*e.g.*, serotypes 12, 18, 31), subgroup B (*e.g.*, serotypes 3, 7, 11, 14, 16, 21, 34, 35), subgroup C (*e.g.*, serotypes 1, 2, 5, 6), subgroup D (*e.g.*, serotypes 8, 9, 10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-47), subgroup E (serotype 4), subgroup F (serotype 40, 41), or any other adenoviral serotype. Preferred serotypes are adenovirus serotypes 2(Ad2), 5 (Ad5) and 35 (Ad35).

Thus, as used herein, "adenovirus" and "adenovirus particle" refer to the virus itself or derivatives thereof and cover all serotypes and subtypes and both naturally occurring and recombinant forms, except where indicated otherwise. Preferably, such adenoviruses are ones that infect human cells. Such adenoviruses may be wildtype or may be modified in various ways known in the art or as disclosed herein. Such modifications include modifications to the adenovirus genome that is packaged in the particle in order to make an infectious virus. Such modifications include deletions known in the art, such as deletions in one or more of the E1a, E1b, E2a, E2b, E3, or E4 coding regions. Such modifications also include deletions of all of the coding regions of the adenoviral genome. Such adenoviruses are known as "gutless" adenoviruses. The terms also include replication-conditional adenoviruses; that is, viruses that preferentially replicate in certain types of cells or tissues but to a lesser degree or not at all in other types. In a preferred embodiment of the invention, the adenoviral particles replicate in abnormally proliferating tissue, such as solid tumors and other neoplasms. These include the viruses disclosed in EP0514603 U.S. Patent Nos. 5,677,178, 5,698,443, 5,871,726, 5,801,029, 5,998,205, 6,432,700, U6254862 and PCT publications WO 97/01358, WO 98/39464, WO 98/39465, WO 00/15820, WO 00/39319, WO 96/34969, WO 97/04805, WO 01/36650, , WO 00/67576, WO 01/23004, and WO 01/04282. Such viruses are sometimes referred to as "cytolytic" or "cytopathic" viruses (or vectors), and, if they have such an effect on neoplastic cells, are referred to as "oncolytic" viruses (or vectors).

The terms "adenovirus vector" and "adenoviral vector" are used interchangeably and are well understood in the art to mean a polynucleotide comprising all or a portion of an adenovirus genome. An adenoviral vector of this invention may be in any of several forms, including, but not limited to, naked DNA, DNA encapsulated in an adenovirus capsid, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA

complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, complexed with compounds such as PEG to immunologically "mask" the molecule and/or increase half-life, or conjugated to a non-viral protein.

As used herein, the terms "vector," "polynucleotide vector," "polynucleotide vector construct," "nucleic acid vector construct," and "vector construct" are used interchangeably herein to mean any nucleic acid construct for gene transfer, as understood by those skilled in the art.

As used herein, the term "viral vector" is used according to its art-recognized meaning. It refers to a nucleic acid vector construct that includes at least one element of viral origin and may be packaged into a viral vector particle. The viral vector particles may be utilized for the purpose of transferring DNA, RNA or other nucleic acids into cells either *in vitro* or *in vivo*. Viral vectors include, but are not limited to, retroviral vectors, vaccinia vectors, lentiviral vectors, herpes virus vectors (*e.g.*, HSV), baculoviral vectors, cytomegalovirus (CMV) vectors, papillomavirus vectors, simian virus (SV40) vectors, Sindbis virus vectors, semliki forest virus vectors, phage vectors, adenoviral vectors, and adeno-associated viral (AAV) vectors. For purposes of the present invention, the viral vector is preferably an adenoviral vector.

The terms "virus," "viral particle," "vector particle," "viral vector particle," and "virion" are used interchangeably and are to be understood broadly as meaning infectious viral particles that are formed when, *e.g.*, a viral vector of the invention is transduced into an appropriate cell or cell line for the generation of infectious particles. Viral particles according to the invention may be utilized for the purpose of transferring nucleic acids into cells either *in vitro* or *in vivo*. For purposes of the present invention, these terms preferably refer to adenoviruses, including recombinant adenoviruses formed when an adenoviral vector of the invention is encapsulated in an adenovirus capsid.

As used herein, the term "Replication Competent Virus" or "RCV" is understood broadly as meaning any virus in a viral preparation that does not retain the same selective replication pattern as the original (parental) selectively-replicating virus. This includes a virus that does not retain the same selective replication pattern as the originally constructed selectively-replicating virus. For example, the originally constructed virus has mutated, so that viruses in the preparation are different from the parental virus and the different virus has a replication selectivity profile different from

the parental virus. An RCV may be of the same type as the parental virus (e.g. both the parental virus and the RCV are based on adenoviruses) or may be different types (e.g. the parental virus is based on a herpes virus and the RCV is an adenovirus).

The term "Replication Competent Adenovirus" or "RCA" is an RCV that is based on any adenovirus.

The terms "selectively-replicating virus", "replication-conditional" "conditionally replicating", "replication-selective" and "replication restricted viruses" are use interchangeably and understood to mean any virus that has been designed to selectively replicate in a type or types of cells. It is not meant to encompass standard replication defective viruses that are not used as replicating viruses, except for the purpose of amplifying more virus. Replication defective viruses usually have a certain function mutated or deleted, which is meant to eliminate or substantially reduce replication of the virus in the cells that are to be infected. Although, deletion of a viral function that may reduce viral replication in a cell, but is intended to allow replication in another cell type would be by definition and is still considered herein as selectively replicating. For example, the adenovirus Addl1520 has the E1b region deleted, but is being used as a selectively-replicating virus for killing tumor (cancer) cells (WO 94/189992; Ganly, et al., "A Phase I Study of Onyx-015, an E1B Attenuated Adenovirus, Administered Intratumorally to Patients with Recurrent Head and Neck Cancer," Clinical Cancer Research, 6:798-806 (March 2000)).

"Tumor-specific", "tumor-selective", "tumor-selective replication-conditional" or "Tumor-specific replication-conditional" viruses are understood to be selectively-replicating viruses that preferentially replicate in at least one tumor cell type as compared to another cell type. One type of tumor specific virus uses tumor-selective regulatory elements to control the expression of early viral genes essential for replication. (See, e.g., WO 96/17053, WO 99/25860, WO 02/067861, WO 02/068627, WO9701358, WO9839464, WO9839465, WO0015820, WO0039319, WO9634969, WO0136650, WO0067576, WO0123004 and U.S. Patent Nos. 5,698,443, 5,871,726, 5,998,205, 6,432,700, and US6254862), Such oncolytic viruses will selectively replicate and lyse tumor cells if the gene that is essential for replication is under the control of a promoter or other transcriptional regulatory element that is tumor-selective.

Other tumor-specific replication-conditional adenoviruses have one or more mutations or deletions in the E1a and/or E1b genes such that the E1a protein lacks the capacity to bind RB and such that the E1b protein lacks the capacity to bind p53. (See, e.g., U.S. Pat. No. 5,677,178.)

The term "gene essential for replication" refers to a nucleic acid sequence whose transcription is required for a viral vector to replicate in a target cell. For example, in an adenoviral vector of the invention, a gene essential for replication may be one or more of the E1a, E1b, E2a, E2b, or E4 genes, such as the E1a gene or the E1a and E4 genes.

"Regulatory elements" are sequences involved in controlling the expression of a nucleotide sequence. Regulatory elements include promoters, enhancers, and termination signals. They also typically encompass sequences required for proper translation of the nucleotide sequence.

The term "promoter" refers to an untranslated DNA sequence usually located upstream of the coding region that contains the binding site for RNA polymerase II and initiates transcription of the DNA. The promoter region may also include other elements that act as regulators of gene expression.

Promoters and other transcriptional regulatory elements that are tumor-selective include, but are not limited to, an E2F responsive promoter such as the E2F-1 promoter, a human telomerase reverse transcriptase (hTERT) promoter, an osteocalcin promoter, a carcinoembryonic antigen (CEA) promoter, a DF3 promoter, an α-fetoprotein promoter, an ErbB2 promoter, a surfactant promoter, a tyrosinase promoter, a MUC1/DF3 promoter, a TK promoter, a p21 promoter, a cyclin promoter, an HKLK2 promoter, a uPA promoter, a HER-2neu promoter, a prostate specific antigen (PSA) promoter, a probasin promoter, a glandular kallikrein transcriptional regulatory element (U.S. Patent Application Publication No. US 2002/0136707 A1), a uroplakin II-derived transcriptional regulatory element (U.S. Patent Application Publication No. US 2002/0120117 A1), and a melanoma cell specific transcriptional regulatory element (TRE), e.g. a tyrosinase, tyrosinase-related protein 2 (TRP2), MIA, microphthalmia associated transcription factor (MITF); melanocyte-specific gene 1; melanocyte-specific tyrosinase-related protein-1, or MART-1 derived TRE (all disclosed in U.S. Patent Application Publication No. US 2003/0039633 A1). Also, see, e.g., WO 96/17053, WO 98/13508, WO 98/14593, WO 99/25860, WO

00/46355, WO 02/067861, WO 02/068627, and U.S. Pat. Nos. 5,648,478 and 6,495,130.

As used herein, the terms "cancer," "cancer cells," "neoplastic cells," "neoplasia," "tumor," and "tumor cells" (used interchangeably) refer to cells that exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation. Neoplastic cells can be malignant or benign.

The terms "coding sequence" and "coding region" refer to a nucleic acid sequence that is transcribed into RNA such as mRNA, rRNA, tRNA, snRNA, sense RNA or antisense RNA. Preferably the RNA is then translated in a cell to produce a protein.

The term "expression" refers to the transcription and/or translation of an endogenous gene or a transgene in a cell. In the case of an antisense construct, expression may refer to the transcription of the antisense DNA only.

The term "gene" refers to a defined region that is located within a genome and that, in addition to the aforementioned coding sequence, comprises other, primarily regulatory, nucleic acid sequences responsible for the control of expression, *i.e.*, transcription and translation of the coding portion. A gene may also comprise other 5' and 3' untranslated sequences and termination sequences. Depending on the source of the gene, further elements that may be present are, for example, introns.

The terms "heterologous" and "exogenous" as used herein with reference to nucleic acid molecules such as promoters and gene coding sequences, refer to sequences that originate from a source foreign to a particular virus or host cell or, if from the same source, are modified from their original form. Thus, a heterologous gene in a virus or cell includes a gene that is endogenous to the particular virus or cell but has been modified through, for example, codon optimization. The terms also includes non-naturally occurring multiple copies of a naturally occurring nucleic acid sequence. Thus, the terms refer to a nucleic acid segment that is foreign or heterologous to the virus or cell, or homologous to the virus or cell but in a position within the host viral or cellular genome in which it is not ordinarily found.

The term "homologous" as used herein with reference to a nucleic acid molecule refers to a nucleic acid sequence naturally associated with a host virus or cell.

The term "native" refers to a gene that is present in the genome of wildtype virus or cell.

The term "naturally occurring" or "wildtype" is used to describe an object that can be found in nature as distinct from being artificially produced by man. For example, a protein or nucleotide sequence present in an organism (including a virus), which can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory, is naturally occurring.

The term "nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof ("polynucleotides") in either single- or double-stranded form. Unless specifically limited, the term encompasses nucleic acids containing known analogues of natural nucleotides that have similar binding properties as the reference nucleic acid and are metabolized in a manner similar to naturally occurring nucleotides. Unless otherwise indicated, a particular nucleic acid molecule/polynucleotide also implicitly encompasses conservatively modified variants thereof (*e.g.* degenerate codon substitutions) and complementary sequences and as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, Nucleic Acid Res. 19: 5081 (1991); Ohtsuka *et al.*, J. Biol. Chem. 260: 2605-2608 (1985); Rossolini *et al.*, Mol. Cell. Probes 8: 91-98 (1994)). In the context of the present invention, the nucleic acid molecule/polynucleotide is preferably a segment of DNA. Nucleotides are indicated by their bases by the following standard abbreviations: adenine (A), cytosine (C), thymine (T), and guanine (G).

A nucleic acid sequence is "operatively linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or regulatory DNA sequence is said to be "operatively linked" to a DNA sequence that codes for an RNA or a protein if the two sequences are operatively linked, or situated such that the promoter or regulatory DNA sequence affects the expression level of the coding or structural DNA sequence. Operatively linked DNA sequences are typically, but not necessarily, contiguous.

The term "ORF" means Open Reading Frame.

The term "Ar5" refers to the virus Ar5pAE2fF which is shown in Figure 10. Ar5pAE2fF is the same as Ar6pAE2fF except that the packaging sequence is located on the "left" end of the viral genome.

The virus Ar6pAE2fF is described in PCT/US02/05300 (WO02/067861)

"OAV" and "Ar6pAE2fE3F" are used interchangeably and are described in detail in PCT/US02/05280 (WO 02/068627) and PCT/US02/05300 (WO 02/067861), and is a genetically modified human adenovirus designed to preferentially replicate in tumor cells, resulting in tumor cell death due to oncolysis. Ar6pAE2fE3F utilizes the E2F-1 promoter to control the expression of E1a. Selective activation of the E2F-1 promoter in tumor cells is based on the repression of E2F-1 promoter activation in normal cells and both the derepression and activation of the E2F-1 promoter in tumor cells. Several reports indicate that defects in the Rb-pathway in tumor cells lead to elimination of cellular factors necessary to repress transcription from the E2F-1 promoter. These defects also lead to an accumulation of "free" E2F-1 that in turn activates the E2F-1 promoter. The majority of cancers are expected to have defects in the Rb-pathway either through mutation in the Rb gene itself, or in factors up stream or down stream of Rb. Thus, Ar6pAE2fE3F is expected to preferentially replicate and kill tumor cells that have defects in the Rb-pathway as compared to cells with an intact Rb-pathway, *i.e.* normal cells.

Ar17pAE2fFTrtex, which is described in detail in PCT/US02/05300 (WO 02/067861), is a tumor-selective oncolytic adenovirus designed to be delivered systemically for the treatment of a broad range of cancer indications. The replication of Ar17pAE2fFTrtex is engineered to be dependent on the presence of the two most common alterations in human cancer, namely defects in the Rb-pathway (~85% of all cancers) and overexpression of telomerase (~85% of all cancers). As with Ar6pAE2fE3F, Ar17pAE2fFTrtex utilizes the E2F-1 promoter to control expression of the adenoviral E1a gene. To increase tumor selectivity appropriate for systemic delivery, the adenoviral E4 gene in Ar17pAE2fFTrtex is controlled by the hTERT (human telomerase reverse transcriptase) promoter. Ar17pAE2fFTrtex is expected to replicate in the majority of cancer cells, leading to tumor selective-expression of toxic viral proteins, cytolysis, and enhancement of sensitivity to chemotherapy, cytokines and cytotoxic T lymphocytes.

In another embodiment, an adenovirus produced according to the invention further comprises at least one heterologous coding sequence, such as a therapeutic

gene coding sequence. The therapeutic gene coding sequence, for example in the form of cDNA, can be inserted in any position that does not adversely affect the infectivity or replication of the virus. Preferably, it is inserted in the E3 region in place of at least one of the polynucleotide sequences coding for the E3 proteins. For example, the therapeutic gene coding sequence may be inserted in place of the 19kD or 14.7 kD E3 gene.

A therapeutic gene coding sequence can be one that exerts its effect at the level of RNA or protein. Therapeutic gene coding sequences that may be introduced into the adenovirus include a factor capable of initiating apoptosis, antisense or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, transcription factors, polymerases, etc., sequences encoding cytotoxic proteins, sequences that encode an engineered cytoplasmic variant of a nuclease (*e.g.* RNase A) or protease (*e.g.* trypsin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like.

Other therapeutic genes of interest include, but are not limited to, immunostimulatory, anti-angiogenic, and suicide genes. Immunostimulatory genes include, but are not limited to, genes that encode cytokines (GM-CSF, IL1, IL2, IL4, IL5, IFNα, IFNγ, TNFα, IL12, IL18, and flt3), proteins that stimulate interactions with immune cells (B7, CD28, MHC class I, MHC class II, TAPs), tumor-associated antigens (immunogenic sequences from MART-1, gp100(pmel-17), tyrosinase, tyrosinase-related protein 1, tyrosinase-related protein 2, melanocyte-stimulating hormone receptor, MAGE1, MAGE2, MAGE3, MAGE12, BAGE, GAGE, NY-ESO-1, β-catenin, MUM-1, CDK-4, caspase 8, KIA 0205, HLA-A2R1701, α-fetoprotein, telomerase catalytic protein, G-250, MUC-1, carcinoembryonic protein, p53, Her2/neu, triosephosphate isomerase, CDC-27, LDLR-FUT, telomerase reverse transcriptase, and PSMA), cDNAs of antibodies that block inhibitory signals (CTLA4 blockade), chemokines (MIP1α, MIP3α, CCR7 ligand, and calreticulin), and other proteins. Anti-angiogenic genes include, but are not limited to, genes that encode METH-1, METH -2, TrpRS fragments, proliferin-related protein, prolactin fragment, PEDF, vasostatin, various fragments of extracellular matrix proteins and growth factor/cytokine inhibitors. Various fragments of extracellular matrix proteins include, but are not limited to, angiostatin, endostatin, kininostatin, fibrinogen-E fragment, thrombospondin, tumstatin, canstatin, and restin. Growth factor/cytokine inhibitors

include, but are not limited to, VEGF/VEGFR antagonist, sFlt-1, sFlk, sNRP1, angiopoietin/tie antagonist, sTie-2, chemokines (IP-10, PF-4, Gro-beta, IFN-gamma (Mig), IFNα, FGF/FGFR antagonist (sFGFR), Ephrin/Eph antagonist (sEphB4 and sephrinB2), PDGF, TGFβ and IGF-1.

A "suicide gene" encodes a protein that itself can lead to cell death, as with expression of diphtheria toxin A, or the expression of the protein can render cells selectively sensitive to certain drugs, *e.g.*, expression of the Herpes simplex thymidine kinase gene (HSV-TK) renders cells sensitive to antiviral compounds, such as acyclovir, gancyclovir and FIAU (1-(2-deoxy-2-fluoro-β-D-arabinofuranosil)-5-iodouracil). Other suicide genes include, but are not limited to, genes that encode carboxypeptidase G2 (CPG2), carboxylesterase (CA), cytosine deaminase (CD), cytochrome P450 (cyt-450), deoxycytidine kinase (dCK), nitroreductase (NR), purine nucleoside phosphorylase (PNP), thymidine phosphorylase (TP), varicella zoster virus thymidine kinase (VZV-TK), and xanthine-guanine phosphoribosyl transferase (XGPRT). Alternatively, the therapeutic gene can exert its effect at the level of RNA, for instance, by encoding an antisense message or ribozyme, a protein that affects splicing or 3' processing (*e.g.*, polyadenylation), or a protein that affects the level of expression of another gene within the cell, *e.g.* by mediating an altered rate of mRNA accumulation, an alteration of mRNA transport, and/or a change in post-transcriptional regulation. The addition of a therapeutic gene to the virus results in a virus with an additional antitumor mechanism of action. Thus, a single entity (*i.e.*, the virus carrying a therapeutic transgene) is capable of inducing multiple antitumor mechanisms.

Alternately, the therapeutic gene coding sequence encodes thymidine kinase, Nos, FasL, sFasR (soluble Fas receptor), or granulocyte macrophage colony stimulating factor (GM-CSF; U.S. Patent No. 5,908,763).

The therapeutic gene coding sequence is under the control of a suitable promoter. Suitable promoters that may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter and/or the E3 promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the Rous Sarcoma Virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin promoter;

the ApoAI promoter; and a tissue-selective promoter such as those disclosed in PCT/EP98/07380 (WO 99/25860).

The invention further contemplates combinations of two or more transgenes with synergistic, complementary and/or nonoverlapping toxicities and methods of action. The resulting oncolytic adenovirus would retain the viral oncolytic functions and would, for example, additionally have the ability to induce immune and anti-angiogenic responses, etc.

In another embodiment, adenoviruses produced according to the invention further comprise a targeting ligand included in a capsid protein of the particle. In one embodiment, the capsid protein is a fiber protein and the ligand is in the HI loop of the fiber protein. The adenoviral vector particle may also include other mutations to the fiber protein. Examples of these mutations include, but are not limited to those described in US application no. 10/351,890, WO 98/07877, WO 01/92299, and US Patent Nos. 5,962,311, 6,153,435, and 6,455,314. These include, but are not limited to mutations that decrease binding of the viral vector particle to a particular cell type or more than one cell type, enhance the binding of the viral vector particle to a particular cell type or more than one cell type and/or reduce the immune response to the adenoviral vector particle in an animal. In addition, the adenoviral vector particles produced according to the invention may also contain mutations to other viral capsid proteins. Examples of these mutations include, but are not limited to those described in US Patent Nos. 5,731,190, 6,127,525, and 5,922,315. Other adenoviruses that can be produced according to the invention are described in U.S. Patent Nos. 6,057,155, 5,543,328 and 5,756,086.

Viruses are made by transferring vectors into packaging cells by techniques known to those skilled in the art. Packaging cells may complement functions deleted from the wild-type virus genome. The production of such particles requires that the vector be replicated and that those proteins necessary for assembling an infectious virus be produced. The packaging cells are cultured under conditions that permit the production of the desired viral particle. The particles are recovered by standard techniques.

Selectively replicating viral vectors are promising candidates for our arsenal to treat cancer including metastatic disease. A biological assay is described herein for unintended recombinant adenoviruses which are no longer replication-selective, but rather replicate non-selectively much like parental wild-type viruses. This biological

assay or a variation thereof is broadly applicable to selectively replicating viral products. A biological assay has a distinct advantage in testing for replication competent virus (as is well illustrated in the examples below by the discovery of the REC133 RCV and related RCVs in the Ar6pAE2fE3F vector particle preparation). The biological assays of the present invention are capable of detecting recombinants due not only to predicted but also due to unpredicted recombination events. As described in detail in the examples, the assay detected RCV's that are not predicted (e.g. REC133) and would not have been detected by PCR assays that would have been developed in the anticipation of predicted recombinants (e.g. between the vector and packaging sequences).

In one embodiment the biological assay for RCV can be considered to have two phases. The first phase is the relative amplification of the RCV compared to the intended selectively-replication virus by passaging the viral sample on the cell line (e.g. MRC-5 in the case of Ar6pAE2fE3F as described in the examples). The second phase includes all appropriate analyses of the amplified material in order to detect and confirm the presence of an RCV Increased CPE, increased virus production, increased potency to kill normal cells, altered restriction digest patterns or other means of characterization of the virus may be used in any combination as detection methods to indicate the presence or absence of an RCV that is relatively amplified in the cells selected for the assay. In the case of the assay developed for Ar6pAE2fE3F, the MRC-5 cells do allow some replication of the Ar6pAE2fE3F virus and some CPE is observed at the time of optimum harvest. The CPE observed can be variable, and 'scoring' it is subjective. This does not exclude that assays developed for other selectively-replicating viruses may be able to use CPE as observable indication of RCV. Parameters may be optimized whereas selectively-replicating viruses only develop CPE (or an observable difference in CPE) when an RCV is present at what is considered unacceptable amounts.

The RCV detection assays of the invention are not meant to detect every virus present in a preparation that has even a slightly altered pattern of selectivity or is present in acceptable amounts, but to detect RCVs that may pose problems for the desired purpose of the viral preparation. Acceptable amounts is meant to be an amount that does not significantly effect the intended use of the virus.

For example, in the case of in vitro killing of cancerous cells in a population of non-cancerous cells the purpose may be obtaining a culture free of cancerous cells,

although the death of some noncancerous cells is considered acceptable. Therefore, in this case a contaminating virus present in the preparation that has only a slight change in selectivity that would cause the killing of a limited amount of noncancerous cells would be acceptable. Also, in the example a low level contamination of a potent RCV maybe acceptable. For instance, if the initial input virus is only $1x10^5$ virus particles of the selectively-replicating per experiment and the level contamination is found to be at a level of less than 1 RCV in $1x10^8$ total virus particles, then the level of contamination would probably be considered acceptable, since theoretically less than 0.1% of the experiments would ever be compromised by the contaminating RCV.

The sensitivity to detect RCV will likely depend on both the nature of the replication-selective virus and that of the RCV. A greater difference in their relative selectivity should allow a greater sensitivity of detection by these methods.

Another read out for the assays of the invention is total viral particle production. In some cases, as compared across passages total viral particle production may be a more quantitative and robust indicator. The cytotoxicity potency assay is another very useful indicator of amplified RCV, and in some cases may be more reliable than even viral particle production. Restriction digests provide the most concrete visual indication of altered structure, but are dependent upon: (1) selecting a restriction endonuclease that yields a detectably different restriction pattern for the recombinant, and (2) amplification of the recombinant to a frequency high enough that it is detected by this relatively insensitive method. Banding patterns may be misleading if an aberrant fragment is not adequately resolved from expected fragments. As described in the examples below, the REC133 recombinants in Ar6pAE2fE3F amplified to more than 10% of the total genomes. However, they were resolved with only one of the five endonucleases chosen. Another read out in vivo toxicity. For example, both the original virus preparation (P0) and a preparation from assay amplification (e.g., P4) can be injected in an animal in vivo and various data collected and compared. For example, inject the virus intravenously into mice and measure parameters of toxicity. For example, serum may be collected at various time points after injection and liver enzyme measured, which may be indicators of hepatotoxicity. Increased hepatotoxicity in the amplified preparation (e.g., P4) as compared to the original (P0) preparation may indicate the presence of an RCV. The use of multiple detection methods for an RCA Bioamplification Assay of the present

invention is contemplated and in fact will probably increase the sensitivity of the assay.

Analysis of the amplified material can be extended to include some characterization of the recombinants detected. One concern with a biological amplification assay such as this is that a previously absent recombinant might be generated during the amplification on MRC-5 cells. Thus, it may be desirable to clone out the RCV(s) and develop a method for detecting the RCV(s). For example, the method of detection may be based on PCR. In the examples below, a developed PCR assay for the identified recombinant sequence REC133 is able to confirm that the recombinant RCA was indeed present in the initial (P0) Ar6pAE2fE3F material at a frequency exceeding 1 copy in 1 x $10^7$ vp. In contrast, Ar5pAE2fF that is cloned and produced on HeLa-S3 cells shows no change in any of the read-out methods, indicating that no RCV is present in the starting material.

The disclosure presented herein describes a biological assay for detection of RCV in selectively-replicating virus products, and that these products may contain RCV's with genomes that cannot be predicted. A biological assay such as this that permits differential amplification of a RCV and the selectively-replicating virus is of general utility for testing selectively-replicating viral products, regardless of the arrangement of the virus genome.

As shown below in the examples, a Bioamplification Assay for Replication-Selective Vectors has been successfully developed to detect RCV in a preparation of selectively-replicating virus. The examples describe the assay for detecting RCV in an adenoviral preparation, but the teachings described herein are not limited to an assay to detect RCAs or to performing the assay on only adenoviral preparations.

## EXAMPLES

The present invention is described by reference to the following Examples, which are offered by way of illustration and are not intended to limit the invention in any manner. Standard techniques well known in the art or the techniques specifically described below are utilized.

**EXAMPLE 1: Cell culture and virus production.**

The tumor cell line derivative HeLa-S3 and the normal diploid human fibroblast cell lines MRC-5, Hs68 and WI-38 are obtained from American Type Culture Collection (Manassas, VA). MRC-5 and WI-38 cells are cultured in EMEM containing 10% fetal bovine serum (10% EMEM). HeLa-S3 and Hs68 cells are cultured in DMEM supplemented with 10% fetal bovine serum (FBS). Small airway epithelial cells (SAEC), bronchial epithelial cells (NHBE), renal epithelial cells (HRE), mammary epithelial cells (HMEC), aortic endothelial cells (HAEC), microvascular endothelial cells (HMVEC), lung fibroblasts (NHLF), and prostate epithelial cells (PrEC) are all primary human cells obtained from Clonetics/Biowhittaker (Walkersville, MD), and are cultured in the manufacturer's cell type-specific media. AE1-2A is an adenoviral vector complementing cell line derived from A549 {Gorziglia, Kadan, et al. 1996}, and is cultured in Richter's medium containing 10% FBS. PER.C6 is also an adenoviral vector complementing cell line (Crucell, Leiden, The Netherlands; {Fallaux, Bout, et al. 1998}) and is cultured in DMEM supplemented with 10% FBS and 10 mM MgCl2. All cells are cultured at 37°C and 5% CO2, except Hs68, HeLa-S3 and PER.C6 cells, which are cultured at 37°C and 10% CO2.

Adenoviral vector particles are produced in the indicated cell line and purified on CsCl gradients using methods described previously {Jakubczak, Rollence, et al.} {Jakubczak, Ryan, et al. 2002}.

**EXAMPLE 2: Limiting dilution cloning of virus.**

Thirty-one 96-well tissue culture plates are seeded with 5 x $10^3$ PER.C6, MRC-5 or HeLa-S3 cells/well as indicated, in 100 µl/well. For a given virus to be cloned in a given cell line, three dilutions of the viral vector particles are prepared and added to 10 plates/dilution, in a volume of 100 µl/well. The three dilutions of viral vector particles are chosen such that the middle dilution is calculated to yield ~3

positive wells per 10 plates (e.g. an infecting concentration of 0.03 pfu/ml), with the other two dilutions differing by 10-fold higher and lower. The 31st plate for each vector is infected with higher concentrations of the viral vector particles to serve as positive controls for observable CPE. The virus-infected cells are incubated for 10-14 days and then scored for CPE. Whenever cloning on HeLa-S3 and sometimes when cloning on PER.C6, scoring of positive wells is achieved with a secondary infection of S8 cells. In these cases, S8 cells are seeded on 96-well tissue culture plates at 5 x $10^4$ cells/well in Richter's medium with 10% FBS, 2 mM glutamine, and 0.5 μM dexamethasone added (100 μl/well). Secondary infection is performed after 1 freeze and thaw of the primary infection plates, by transferring 50 μl of the contents of each well of the primary infection plates to the corresponding well of the S8 plates. The primary infection plates are refrozen and stored at -80°C. After three days, the secondary infection plates are observed for CPE. Primary infection plates corresponding to secondary infection plates containing CPE-positive wells are thawed and the clonal isolates in the primary infection plates are then amplified, preferably in the same cell line in which they had been cloned, first in 6-well plates and then in T-175 flasks. Crude viral lysates (CVL's) are prepared from the T-175 flasks and the clonal isolates are analyzed by restriction digest and agarose gel electrophoresis. Based on the restriction digest analysis of genome structure, selected clones are chosen for production of purified vector particle lots and further analysis.

This example is one of many methods to clone a virus by limiting dilution (e,g. plaque purification) that are known to one skilled in the art.

**EXAMPLE 3: Secondary hexon titer assay.**

The secondary hexon titer assay is an indirect measure of adenoviral titer. Samples are applied to a cell line, AE1-2A, that is permissive to viral vector replication. After 3 days, the cells are analyzed by an ELISA for total adenoviral hexon produced. The hexon signal is proportional to the input virus titer. The titer of unknown samples is determined by generating a standard curve using dilutions of a reference standard vector.

AE1-2A cells are seeded into 96-well tissue culture plates on the day of infection at 5 x $10^4$ cells/well (in 100 μl), in Richter's medium supplemented with 10% FBS, 2 mM glutamine, and 1 μM dexamethasone. Only the inner 60 wells of

each plate are seeded with cells, while the outer wells of each plate are filled with 200 µl DPBS. Serial dilutions of standards and unknowns are prepared (1:2 for known concentrations of Ad5 and Ar6pAE2fE3F to serve as the standard curves and 1:4 for each unknown production sample) in the same medium. Secondary infection of the AE1-2A cells takes place by adding 100 µl of each dilution of production sample or standard curve to triplicate wells of AE1-2A cells and incubating the plates for three days at 37°C with 5% CO2, with no further media changes. After three days, the secondary infection plates are harvested and three freeze-thaws are performed.

An ELISA is performed on these samples by first coating the inner 60 wells of the appropriate number of Immulon 96-well flat-bottom plates. Plates are coated overnight at 4°C with 100 µl anti-hexon purified antibody (2.5 µg/ml; Chemicon #MAB8052, diluted in 20 mM Tris + 150 mM NaCl). The next day, the plates are washed three times (20 mM Tris, 150 mM NaCl, 0.05% Tween 20), blocked with Pierce SuperBlock (Pierce Cat #37545), and washed again. CVLs from the secondary infection plates are diluted 1:500 with dilution buffer (20 mM Tris, 150 mM NaCl, 1% bovine albumin fraction V), and 100 µl of each diluted lysate is added to the pre-coated wells. The plates are incubated at room temperature (R.T.) for 1 hour, followed by washing as above. Anti-hexon biotinylated antibody (2.5 µg/ml, diluted in dilution buffer) is then added to each well (100 µl/well) and the plates are incubated at room temperature (R.T.) for 1 hour. After incubation, plates are washed and 100 µl Streptavidin alkaline phosphatase (1:2000 dilution of 1 mg/ml stock solution; Pierce Cat #21324) is added to each well. After a 30 min incubation at R.T., the plates are washed and pNPP substrate (p-Nitrophenyl Phosphate, Disodium; Sigma #N-2770) is added to each well (100 µl/well). The plates are incubated at R.T. for 1 hour. Absorbance is read on a microplate reader at a test wavelength of 405 nm while subtracting the absorbance at the reference wavelength of 490 nm. Standard curves and sample data are analyzed using GraphPad Prism.

**EXAMPLE 4: Generation of an Ar6pAE2fE3F preparation**

A research viral seed lot of Ar6pAE2fE3F is generated on AE1-2A cells, a derivative of A549 cells (Gorziglia MI, Kadan MJ, Yei S, Lim J, Lee GM, Luthra R, Trapnell BC, "Elimination of both E1 and E2 from adenovirus vectors further improves prospects for in vivo human gene therapy." J Virol 1996 Jun;70(6):4173-8).

Following limiting dilution cloning on PER.C6 cells, a single clone of Ar6pAE2fE3F is expanded on PER.C6 cells to generate an Accessionary Virus Bank (AcVB). The AcVB is purified by centrifugation on a cesium chloride gradient. Molecular and biologic characterization indicates that the AcVB is acceptable for generation of further viral banks. It should be noted that limiting dilution cloning followed by production of an AcVB may require a significant number of viral passages.

**EXAMPLE 5: Procedure for screening cell lines for differential amplification of "wild-type" versus selectively replicating adenoviruses**

To develop an assay capable of detecting an RCA in a background of replication-selective adenoviuses, normal cell lines are evaluated for increased production of an RCA compared to the selectively-replicating adenovirus, i.e. "relative amplification". Cell lines that may be evaluated include, but are not limited to, the fibroblast cell lines MRC-5, Hs68 and WI-38, as well as primary human Small Airway Epithelial Cells, SAEC (Clonetics). For example, for detecting RCA in a preparation of Ar6pAE2fE3F, a wild-type Ad5 is used to model potential RCA, and the oncolytic virus Ar6pAE2fE3F (Figure 10) is used as the test article. Production tests are performed in 75 $cm^2$ tissue culture flasks, with cells cultured to 50-70% confluence. Multiple multiplicities of infection (0.4-50 particles per cell; ppc) and times of harvest (3-12 days post-infection) are assessed. At harvest, cells are collected, a cell pellet is formed by centrifugation, and a crude viral lysate (CVL) is prepared by: (1) resuspending the cell pellet in a small volume of Dulbecco's phosphate buffered saline (DPBS), (2) three cycles of freeze-thawing, and (3) clarification by low speed centrifugation. Productivity of virus is determined by titering CVL's using a secondary hexon titer assay. For example, for detecting RCA in a preparation of Ar6pAE2fE3F, these initial production tests are performed with wild-type Ad5 and Ar6pAE2fE3F in separate flasks. The goal of these initial experiments is to select conditions for further development using mixtures of Ad5 and and the test article (e.g. Ar6pAE2fE3F).

Criteria used to select a cell line and culture conditions for further development include the relative production of Ad5 wild-type compared to the test article (e.g. Ar6pAE2fE3F), the absolute virus productivity, the general growth characteristics of the cells, and the overall feasibility of assay development. The required virus yield is set at approximately 1 x $10^{12}$ purified viral particles (vp) to enable molecular analyses

such as quantitative PCR, restriction digest mapping and sequencing, if warranted. More or less virus may suffice depending on which diagnostic assays are needed.

**EXAMPLE 6: Screening cell lines and initial development of an RCA bioamplification assay for Ar6pAE2fE3F preparations**

The following are results from when cell lines are screened with a wild-type Ad5 as a model for potential RCA, and the oncolytic virus Ar6pAE2fE3F preparation is used as the test article. The procedure in example 5 is used. Cell lines evaluated include the fibroblast cell lines MRC-5, Hs68 and WI-38, as well as primary human Small Airway Epithelial Cells, SAEC (Clonetics). WI-38 and SAEC were both rejected early due to poor characteristics for growth to large scales required for this assay.

The principle criteria used to select a cell line and culture conditions for further development is the relative production of Ad5 wild-type compared to Ar6pAE2fE3F, the absolute virus productivity, the general growth characteristics of the cells, and the overall feasibility of assay development. The required virus yield is set at approximately $1 \times 10^{12}$ purified viral particles (vp) to enable molecular analyses such as quantitative PCR, restriction digest mapping and sequencing, if warranted. In general, longer times before harvest or greater MOI increase total productivity, but also decrease the difference in the amount of Ad5 produced compared to Ar6pAE2fE3F. Based on these comparisons, MRC-5 cells are selected for assessment of biological amplification of Ad5 spiked into Ar6pAE2fE3F to determine whether this method can be used to detect low levels of RCA in an oncolytic adenoviral virus preparation. The harvest time is set to 6 days post-infection, after infecting with an MOI of 10-50 ppc.

**EXAMPLE 7: Detection of an RCA in a preparation of Ar6pAE2fE3F**

Based on these data from Example 6, MRC5 cells are selected for assessment of biological amplification of Ad5 spiked in Ar6pAE2fE3F. The assay is designed to include 4 passages of virus on MRC5 cells with PCR detection of only 2 predicted classes of potential RCA. These predicted RCAs are based on the potential for recombination between viral vector and packaging sequences in the cell line (Figure 1).

The RCA Bioamplification Assay is initially tested at full scale using Ar6pAE2fE3F, either alone or spiked with wild-type Ad5 at ratios ranging from 1 plaque forming unit (pfu) Ad5 (wild-type) per 3 x $10^{10}$ vp Ar6pAE2fE3F to 1 x $10^{4}$ pfu Ad5 per 3 x $10^{10}$ vp Ar6pAE2fE3F. One viral production passage is defined as including infection of the MRC-5 cells with a known amount of virus, culturing for 6 days with one change of medium, and harvesting the virus produced. Each virus sample is passaged sequentially four times in MRC-5 cells. In the starting sample (P0) and at each subsequent passage (P1-P4) the total number of adenoviral particles present is determined using an OD260/SDS method, and the number of copies of Ad5 present is determined using a quantitative PCR assay specific for the wild-type E1a promoter (absent in Ar6pAE2fE3F). The ratio of Ad5 to total adenoviral particles is compared between each passage to determine whether relative amplification of Ad5 (the model RCA) compared to the test virus is achieved.

In preliminary experiments, relative amplification of Ad5 was consistently observed for samples spiked at 100 pfu Ad5 or greater per 3 x $10^{10}$ vp Ar6pAE2fE3F. Preliminary results with 1 pfu Ad5 per 3 x $10^{10}$ vp Ar6pAE2fE3F were inconclusive due to the presence of an unexpected RCA present in the lot of Ar6pAE2fE3F used, and which is described below.

During the amplification of Ar6pAE2fE3F described above, cytopathic effect (CPE) was observed to be minimal at the time of harvest (6 days) for P1, P2 and P3 in all spiked and unspiked samples. It should be noted that if the cultures were incubated for additional days, CPE was observed to increase. However, at P4 the amount of CPE was noticeably increased at the time of harvest compared to earlier passages. At P1, P2 and P3 it was necessary at harvest to incubate the cells with EDTA in order to release the cells from the plastic substrate. At P4 the cells easily entered suspension with mild shaking of the roller bottles. Moreover, the yield of virus was greater at P4 then at earlier passages. These effects were observed in the Ar6pAE2fE3F samples that were spiked with Ad5, and unexpectedly in unspiked samples. The increase in CPE and increase in virus yield are consistent with a change in the virus composition during passaging on MRC-5 cells, so that a virus or virus mixture less selective for tumor cells and more productive on normal cells was present at P4 than at P0. These observations lead us to suspect the presence of a non-selective RCV in the Ar6pAE2fE3F virus preparation and was later confirmed that an RCA was present in

relatively low levels in the initial adenovirus preparation and the percentage of RCA was amplified at each passage.

The assay was designed to include 4 passages of virus on MRC5 cells with PCR detection of only 2 classes of potential RCA. These predicted RCAs are based on the potential for recombination between virus and packaging plasmid sequences (Figure 1). However, during the development of the amplification portion of the assay, a biological change in the MRC5 cells was observed after the third passage of Ar6pAE2fE3F even though no model RCA had been spiked into the initial sample. The yield of virus increased at passage 3 and by passage 4 an obvious cytopathic effect was observed.

In the description above, MRC5 cells are used for the amplification steps of the assay. The present invention is not meant to be limited to a particular cell line. For example selectively-replicating (e.g. oncolytic) viruses may for the purposes of the assay be passaged ("amplified") on any "normal" or non-target cell type. Normally several of these non-target cell types are screened based on the differential or selective amplification of the RCA as compared to a selectively-replicating virus.

Also, the number of passages on the non-target cells may be increased or decreased to optimize for the differential amplification of the selective replicating virus as compared to an RCA. See example 5.

The "read-out" of the assay may include any of the many factors or combinations thereof including: observation of CPE, measurement of total virus or yield, analysis for molecular structure (e.g. restriction digestion analysis, nucleotide sequencing), measurement of viral potency (e.g. cell killing assays). These factors may be measured/observed at the end of the amplification process or at each passage during this process. Then measurements/observations may be compared to those made from the first passage or starting viral preparation.

**EXAMPLE 8: General procedure for characterizing an RCA in a preparation of adenovirus**

If an RCA is detected the RCA may be molecularly characterized. One way to characterize the RCA is to perform a restriction digest on viral DNA isolated from one of the passages, preferably the last passage, of the assay. If an uncharacteric restriction pattern is displayed, the unexpected fragment is cloned and sequenced.

To determine whether a rearranged virus is present in the AcVB, is generated as a consequence of forced virus production on a poorly permissive cell line or generated by another means, a PCR assay is developed to specifically detect the rearranged viral vector. Preferably, this PCR assay has a sensitivity of at least 10-100 rearranged vector in $10^8$ viral vectors. Using this assay, viral preparations are tested.

**EXAMPLE 9: Characterization of an RCA in a preparation of Ar6pAE2fE3F**

The following describes a procedure that may be used to characterize the viral DNA from passage 7 of example 2. Diagnostics of the passage 4 virus particles revealed a restriction pattern that is not characteristic of Ar6pAE2fE3F.

The unexpected fragment is cloned and sequenced. The sequence analysis suggests that viral vector rearrangement has occurred resulting in a loss of most of the E2F-1 promoter and replacement with most of the viral E4 promoter (Figure 5) such that the predicted tumor selectivity of E1a expression and viral replication are lost. Surprisingly, the assay detected an RCA that was not predicted by the inventors and therefore would not have been detected by the PCR designed to detect the two classes of predicted RCA (Figure 1).

To determine whether the rearranged virus is present in the AcVB, a PCR assay is developed to specifically detect the rearranged vector. Preferably, this PCR assay has a sensitivity of at least 10-100 rearranged viral vectors in $10^8$ viral vectors. Using this assay, the AcVB, Master Virus Bank (MVB) and Pre-Clinical Material are tested. When performed on the above mentioned lots of Ar6pAE2fE3F all of these virus banks and product lots are positive at levels between 30-200 RCA vectors per $10^8$ vector particles (Table 1). Further, these results show that passaging of a replication-selective virus in the less permissive cells such as MRC-5 does not lead to the generation of RCV, which would be a false positive. The most prevalent RCA has greater cytotoxicity on some normal cells than wild type Ad5 virus (Figures 3 and 6).

**Table 1: Quantitation of Recombinant in Ar6pAE2fE3F by Q-PCR**

| Cell Line | Viral Passage | REC133 Copies[a] per 1 X $10^8$ vp | % REC133 |
|---|---|---|---|
| Production cells | P0[b] | 2.0 X $10^1$ | 0.000020% |
| MRC-5 | P1 | 2.2 X $10^3$ | 0.0022% |
| | P2 | 1.9 X $10^5$ | 0.19% |
| | P3 | 4.5 X $10^6$ | 4.5% |
| | P4 | 4.0 X $10^7$ | 40% |
| Production cells | P2 | 3.4 X $10^1$ | 0.000034% |
| | P4 | 1.6 X $10^2$ | 0.00016% |

[a]PCR results are the average of triplicate determinations.

[b]The initial test material grown in standard production cells is defined as viral passage 0 ("P0"). Subsequent viral passages on either cell line are numbered relative to the initial test material.

Without being bound by theory, the inventors believe that the mechanism of action that leads to the production of these particular RCAs in Ar6pAE2fE3F preparations is as follows. The recombinants probably either are created through intermolecular recombination or by a mechanism of "polymerase jumping." Either one of these mechanisms leads to the viral sequences from the right end of the vector being duplicated, as is seen in the recombinant. This leads to part or all of the tumor selective promoter (E2F-1) being replaced by part or all of the viral promoter for E4. These recombinants could then be packaged and are found to replicate non-selectively or least with a different selectivity as compared to Ar6pAE2fE3F.

In summary, RCAs were detected in preparations of Ar6pAE2fE3F using a cell-based biological assay that involved serially passaging the virus. The RCAs were fortuitously detected while developing the assay. The recombinants that were detected through this assay were unexpected recombinants that were not predicted by the inventors. A PCR-based assay based on predicted recombinants would not have

detected these recombinants. This demonstrates a clear advantage of the cell-based biologic amplification assay over the PCR detection methods for detecting replication competent viruses.

**EXAMPLE 10: Investigation of detected RCA in Ar6pAE2fE3F**

A key characteristic of selectively replicating viruses is decreased potency to kill normal or non-target cells. To help determine whether a non-selective RCA is amplified in the Ar6pAE2fE3F sample, the potencies of the P0 and P4 virus particles to kill normal cells are compared, using a cytotoxicity assay with MTS readout. On MRC-5 cells and on primary Human Aortic Endothelial Cells (HAEC's, Clonetics) the Ar6pAE2fE3F MRC-5 P4 viral particles are more potent than the P0 viral particles by greater than 2 logs (Figures 11 and 12). Surprisingly, the P4 virus is also more potent than wild-type Ad5. A panel of normal primary human cell types are evaluated with varying results ranging from no difference in potency between the P0 and P4 viral particles to a 3+ log increase in potency of the P4 viral particles compared to P0 **(Table 2)**. On no cell type tested is the potency of the P0 viral particles greater than that of the P4 viral particles.

**Table 2: Relative potency of Ar6pAE2fE3F, P4 compared to P0.**

| Cell Line | log(dose ratio) | S.E.M. |
|---|---|---|
| HMEC | 0.23 | 0.082 |
| SAEC | *0.30 | 0.079 |
| NHBE | *0.35 | 0.11 |
| PrEC | *0.57 | 0.11 |
| HRE | *0.90 | 0.17 |
| NHLF | *2.1 | 0.25 |
| HMVEC | *2.6 | 0.23 |
| HAEC | *3.6 | 0.16 |
| MRC-5 | *2.6 | 0.29 |

Log(dose ratio) is calculated as:

$$\log [EC_{50} \text{ of P4}] - \log [EC_{50} \text{ of P0}]$$

*Potency of P4 significantly different from potency of P0, $p < 0.05$, Student's t-test

To further explore whether an RCA is amplified, restriction digest analyses of the P0 and P4 viral vectors are performed. A panel of restriction endonucleases are

selected to provide banding patterns that together might indicate genetic changes in many of the critical regions of the vector. All five digests result in the expected pattern with the exception of SspB1 digestion of the P4 virus, which yields a fragment of unexpected size. The altered potency and restriction digest pattern of the P4 vectors compared to the starting vector strongly suggests that the P4 material contains a significant portion of vector particles with altered molecular structure and decreased replication selectivity. The RCA Bioamplification Assay of Ar6pAE2fE3F virus at P0, including analysis by potency assay and restriction digest, is then repeated in its entirety, which yields equivalent results, confirming the initial finding.

The unexpected restriction fragment of the P4 virus is extracted from the gel, cloned into a plasmid, and sequenced. Sequence analysis of the fragment, and subsequently of cloned recombinant viruses described below, indicates that a rearrangement of the viral vector genome has occurred resulting in a duplication of the right end of the vector in place of the left end. This results in the loss of most or all of the E2F-1 promoter previously driving E1A expression, and replacement with part or all of the adenoviral E4 promoter.

To determine whether the observed recombinant virus is an assay artifact generated during the RCA Bioamplification Assay, or is present in the original test material (Ar6pAE2fE3F at P0), a quantitative PCR assay is developed capable of detecting one of the recombinant sequences, termed "REC133". This PCR assay has a sensitivity of 10 rearranged vector copies in $10^8$ vector particles. PCR analysis for REC133 is performed on Ar6pAE2fE3F virus before and after passaging on MRC-5. REC133 is present in Ar6pAE2fE3F at P0 at a frequency of 20 copies per 1 x $10^8$ vp Ar6pAE2fE3F (Table 3). Thus, the RCA Bioamplification Assay detects a recombinant that was present in the original Ar6pAE2fE3F test sample. The frequency of REC133 increased dramatically with each passage on MRC-5 cells, indicating that "relative amplification" of this non-selective recombinant virus compared to the Ar6pAE2fE3F was achieved as hypothesized in the design of the assay. It was further postulated that in a cell line in which this selective advantage was reduced or eliminated, such as PER.C6, the relative amplification of REC133 would be reduced or absent. To investigate this hypothesis, an aliquot of the original Ar6pAE2fE3F test sample (P0) is passaged sequentially on PER.C6 cells, and the resulting viral lots tested for REC133. The frequency of REC133 increases with each

passage when Ar6pAE2fE3F is passaged in PER.C6 cells, although at a much slower rate than when passaged on MRC-5 cells (Table 3).

**Table 3: Quantitation of REC133 and PGK Recombinants by PCR**

| Vector (Cell line for P0) | Passage[a] on MRC-5 | REC133 Copies[b] Per 1 X $10^8$ vp | % REC133 | PGK Copies[b] per 5 X $10^{10}$ vp | % PGK |
|---|---|---|---|---|---|
| Ar6pAE2fF (PER.C6) | P0 | 3.4 X $10^2$ | 0.00034% | 2.4 X $10^5$ | 0.00048% |
| | P4 | 1.1 X $10^4$ | 0.011% | 5.6 X $10^6$ | 0.011% |
| Ar6pAE2fF (HeLa-S3) | P0 | 4.5 X $10^1$ | 0.000045% | b.d.[c] | 0% |
| | P4 | 3.1 X $10^6$ | 3.1% | b.d. | 0% |
| Ar5pAE2fF (PER.C6) | P0 | b.d.[c] | 0% | 4.0 X $10^6$ | 0.0080% |
| | P4 | b.d. | 0% | 2.8 X $10^9$ | 5.6% |
| Ar5pAE2fF (HeLa-S3) | P0 | b.d. | 0% | b.d. | 0% |
| | P4 | b.d. | 0% | b.d. | 0% |

[a]The initial test material was cloned and produced on the cell line indicated in parentheses below the vector name, and is defined as P0. This lot was then passaged sequentially on MRC-5 cells for four passages.

[b]PCR results are the average of triplicate determinations.

[c]b.d. – below detection. Limit of detection for REC133 is 10 copies per 1 X $10^8$ vp. Limit of detection for PGK promoter sequence is 50 copies per 1 X $10^{10}$ vp.

Ar6pAE2fE3F was originally cloned and produced on PER.C6 cells, which provide E1A to support viral replication. During the RCA Bioamplification Assay REC133 would likely co-infect cells with parental Ar6pAE2fE3F. Therefore it is possible that the recombinant observed is not truly replication competent but is dependent upon E1A provided by either the cells when grown in PER.C6, or helper virus, in this case Ar6pAE2fE3F, when passaged in MRC-5. To more fully characterize the RCA, clonal isolates were prepared from the P4 virus. Because there is no definitive evidence that the recombinant in the P4 virus is replication competent,

limiting dilution cloning is done on both PER.C6 cells and MRC-5 cells to maximize the potential to obtain the recombinant virus. Clones are successfully isolated on both PER.C6 cells and MRC-5 cells. Nineteen clones from PER.C6 and seven from MRC-5 are analyzed by restriction enzyme digest and also by sequencing. The clones isolated include the parental (non-recombined) Ar6pAE2fE3F virus (3 of 26 analyzed). Surprisingly, the restriction digest analyses indicate the presence of multiple recombinants isolated from both PER.C6 and MRC-5 cells, which is confirmed by sequence analysis (Figure 13). Sequencing demonstrates that all of the sequenced RCAs shared the characteristic of a right end duplication to the left end of the vector, with an altered promoter containing all or part of the viral E4 promoter presumably driving E1A expression. In some recombinants a chimeric E4/E2F-1 promoter is present, while in others a complete E4 promoter is present at that position. The clones are also analyzed using the REC133 PCR assay. Only recombinants A and E are detected by this PCR assay, as is predicted from the location of the primers and probe relative to the sequence of each recombinant (Figure 13), reaffirming the limitations of PCR methodology for comprehensive assessment and/or detection of recombinant vectors.

The clonal isolation of some of the recombinants on MRC-5 cells (Figure 13) indicates that at least recombinants A, B, E and F are truly replication competent since MRC-5 cells do not provide any helper function. One additional clone (recombinant C) that is not originally isolated from MRC-5 cells is subsequently shown to replicate on these and other normal cells. Moreover, in those experiments recombinants A, B and C replicate more efficiently than Ad5 in the normal cell lines evaluated (MRC-5, primary human renal epithelial cells, primary human lung diploid fibroblast cells), in agreement with the greater potency of the P4 virus compared to Ad5 in the cytotoxicity assay (Figures 11 and 12). Recombinants D-G are not similarly evaluated for productivity. Because a relatively small number of clones from MRC-5 are analyzed, the lack of cloning of recombinants D and G using MRC-5 cells should not be taken to suggest that they are necessarily replication incompetent or not capable of independent replication.

**EXAMPLE 11: Further Characterization of the RCA Bioamplification Assay**

The preceding results and examples demonstrate that by using an RCA Bioamplification Assay it is possible to detect an RCA in the presence of a

replication-selective adenoviral vector particle preparation. In order to further characterize the assay, the assay is performed using two additional viruses, Ar6pAE2fF and Ar5pAE2fF (Figure 10). Each virus is cloned and produced on two different cell lines, PER.C6 and HeLa-S3. PER.C6 is a derived cell line that expresses the adenoviral E1a gene under the control of the human PGK promoter [{Fallaux, Bout, et al. 1998}]. PER.C6 cells were originally developed to support production of E1-deleted replication defective adenoviral viruses. In the context of selectively-replicating viruses, however, the presence of the E1a sequence preceded by the PGK promoter could allow recombination with the vector to generate a virus with E1a under the control of the PGK promoter, which is constitutive and non-selective [{Murakami, Pungor, et al.}]. The other cell line used for production, HeLa-S3, is a clonal derivative of the tumor cell line HeLa. This transformed cell line supports replication of E2F-1 promoted oncolytic adenoviruses, and so also serves as an excellent production platform for these viruses.

One sample of each virus preparation produced on PER.C6 cells and two samples of each virus preparation produced on HeLa-S3 cells ($4 \times 10^{10}$ vp/sample) are passaged sequentially four times on MRC-5 cells. The degree of CPE increases slightly in the Ar6pAE2fF samples at P3 at the time of harvest while at P4 there is a severe increase in the degree of CPE, regardless of whether the virus is produced on PER.C6 or HeLa-S3 cells. A smaller increase in CPE is observed in the Ar5pAE2fF virus that has been produced on PER.C6 cells. In contrast, an increase in CPE is not observed with the Ar5pAE2fF virus that has been produced on HeLa-S3 cells.

The virus particle concentrations of the purified virus samples are determined by an OD260 method, and the total particles produced at each passage are calculated. The patterns in productivity are similar to the observable patterns of CPE. Productivity increases at P4 with all of the Ar6pAE2fF samples regardless of producer cell platform, and to a smaller degree with the Ar5pAE2fF virus sample that has been cloned and produced in PER.C6 cells. However, no change in productivity during sequential passaging on MRC-5 cells is observed in the Ar5pAE2fF sample that has been cloned and produced in HeLa-S3 cells. Restriction digest analyses are also performed using the same enzymes as are used previously. Aberrant restriction patterns are observed after 4 passages on MRC-5 with Ar6pAE2fF that has been cloned and produced on either PER.C6 or HeLa-S3, and with Ar5pAE2fF that has been cloned and produced on PER.C6. The Ar5pAE2fF sample that is initially cloned

and produced on HeLa-S3 shows the expected restriction digest patterns with all enzymes.

Finally, analysis of potency of the P0 and P4 samples to kill normal cells are determined using MRC-5 cells. The Ar5pAE2fF (HeLa-S3) virus shows no change in potency after four passages on MRC-5 cells (Table 4 and Figure 14). However, the MRC-5 P4 samples of Ar5pAE2fF (PER.C6) and Ar6pAE2fF (PER.C6 or HeLa-S3) all are more potent than the corresponding viral particles at P0. Thus, the observation of CPE and quantitation of production during passaging on MRC-5, potency to kill normal cells before and after passaging on MRC-5 cells, and restriction digest patterns all suggest that Ar5pAE2fF virus that is cloned and produced on HeLa-S3 cells contains no RCA, but that the other 3 virus samples do.

**Table 4: Virus Production During RCA BioAmplification on MRC-5 Cells**

| | Virus (Cell Line for P0) | | | | | |
|---|---|---|---|---|---|---|
| | Ar6pAE2fF (PER.C6) | Ar6pAE2fF (HeLa-S3) | Ar6pAE2fF (HeLa-S3) | Ar5pAE2fF (PER.C6) | Ar5pAE2fF (HeLa-S3) | Ar5pAE2fF (HeLa-S3) |
| P1 | 5.2E+012 | 3.9E+012 | 3.1E+012 | 3.8E+012 | 3.8E+012 | 2.4E+012 |
| P2 | 3.9E+012 | 5.5E+012 | 5.5E+012 | 6.6E+012 | 3.5E+012 | 6.6E+012 |
| P3 | 5.9E+012 | 5.1E+012 | 7.1E+012 | 1.5E+012 | 2.9E+012 | 8.6E+012 |
| P4 | 3.3E+013 | 2.1E+013 | 2.8E+013 | 1.4E+013 | 4.3E+012 | 4.3E+012 |

Each value represents total viral particles purified at viral passage 1-4 (P1, P2...P4) from one experiment.

Murakami and colleagues [{Murakami, Pungor, et al. 2002}] have described adenoviral recombinants that have incorporated the PGK-promoted E1a of the PER.C6 cell line when using that cell line for production of viruses containing overlap with the E1a sequence of the cell line. To partially characterize the recombinants detected by our RCA Bioamplification Assay in the Ar6pAE2fF and Ar5pAE2fF virus preparations, the P0 and P4 viral particles are analyzed by quantitative PCR for both the PGK promoter and REC133. Ar6pAE2fF and Ar5pAE2fF that are originally cloned and produced on PER.C6 cells do contain PGK sequence at moderate frequencies prior to passaging on MRC-5 cells. In both viral preparations, the frequency of PGK sequence in the viral genomes increases after passaging on MRC-5 cells. Neither virus cloned and produced on HeLa-S3 cells contains detectable PGK sequences. Ar6pAE2fF virus that is produced on either PER.C6 or HeLa-S3 cells contain sequences detected by the REC133 PCR. Ar5pAE2fF produced on either cell

line contained no sequences detected by the REC133 PCR. Further characterization was not performed on the PGK promoter-containing recombinant(s) detected in our assay.

Without being bound by theory, molecular structures of the REC133-related recombinants, could have been the result of intermolecular recombination, or intramolecular polymerase jumping. The formation of the REC133-related recombinants may have happened due to at least three structural factors within the Ar6pAE2fE3F and Ar6pAE2fF viruses: (1) the location of the packaging signal on the right end of the molecule; (2) the replacement of the endogenous viral E1 promoter with the human E2F-1 promoter; and (3) the endogenous viral E4 promoter (adjacent to the packaging signal). The lack of detectable RCA in Ar5pAE2fF, in which the packaging signal is located at the left end of the vector genome, suggests that leaving the packaging signal on the left end was sufficient to eliminate similar recombinations in this family of vectors. However, this data should not be interpreted to indicate that placing the packaging signal on the right end in and of itself will lead to the formation of undesirable recombined viruses, nor that leaving the packaging signal on the left end will prevent their formation. Rather, it is the entire vector genome that must be considered, as each element, whether engineered into the vector or an endogenous viral sequence, may influence the nature of any recombinant that is formed. These data suggest that a combination of factors contribute to the generation of RCA in a background of replication-selective virus. The relative importance of each of these factors is likely to vary with each virus construct. An interpretation concerning virus structure to be made from the data shown here is that recombination events can be surprising, thus calling for the use of sequence-independent biological assays such as this one for more comprehensive detection of undesirable recombinants that have formed.

**EXAMPLE 12: A general description of an RCA bioamplification assay for selectively replicating adenovirus preparations on MRC-5 cells.**

The biological assay comprises of four sequential passages of each virus in 20 roller bottles of MRC-5 cells per virus, with CsCl purification of virus at each passage. For each passage, roller bottles (RB) are seeded at 2 x $10^7$ cells/RB and incubated at 37°C with 5% CO2. Two days after seeding, the cells are infected at 50

vp/cell with an estimated cell concentration of 4 x $10^7$ cells/RB (for a total of 4 x $10^{10}$ vp tested per sample). Each roller bottle receives 60 ml infection medium (virus diluted in 10% EMEM). Roller bottles are incubated at 37°C with 5% CO2 for 4 hours and then further filled with 240 ml/RB 10% EMEM supplemented with antibiotics (100 ug/ml streptomycin, 100 U/ml penicillin, 2.5 ug/ml Fungizone; BioWhittaker, Walkersville, MD). Two days after infection, all roller bottles receive a medium change with 300 ml/RB fresh 10% EMEM supplemented with antibiotics.

On Day 6 after infection, cells are harvested. Harvesting the infected cells may be performed by various methods. For example, one method is to aspirate the medium and add 50 ml Versene (0.2 mg/ml EDTA in phosphate buffered saline) to each roller bottle. After 15 to 20 min incubations at 37°C, cells are sharply banged off of the roller bottles and the bottles are rinsed with 10% EMEM. This method is preferred when the cells show lower levels of CPE when infected and incubated in the manner described, and are not easily removed from the surface of the roller bottles. Another harvest method is to simply shake the roller bottles with the medium in them to remove the cells. This method is preferred when the cells show more CPE and/or are already starting to detach from the roller bottles. After harvesting, cells are pelleted by low-speed centrifugation and resuspended in DPBS twice. A crude viral lysate (CVL) is formed by subjecting the cell suspension to 5 freeze-thaw cycles and removing cellular debris by low speed centrifugation. CVL from each virus passage is purified through two rounds of CsCl gradients and dialyzed in THPG dialysis buffer (200 mM Tris, 50 mM HEPES, phosphoric acid to pH 8.0, 10% glycerol) to remove the CsCl.

**EXAMPLE 13: A detailed description of an RCA bioamplification assay for selectively replicating adenovirus preparations on MRC-5 cells.**

**1.0 SEEDING CELLS**

1.1 Trypsinize 2 10-stack cell factories of MRC-5 (ATCC #CCL-171) cells that are ready to be split. Combine the cell suspensions, and count the cells per mL of this suspension.

1.2 Determine the volume of cell suspension required for each roller bottle to be seeded at 2x$10^7$ cells/RB.

1.3 Label the roller bottles with the cell line information (name, passage #, population doubling level, concentration of cells/RB, and date) and add approximately 200mL culture medium (EMEM + 10% FBS) to each bottle.

NOTE: MRC-5 cells have a finite life span and should not be used at the point where the growth starts to decline. ATCC estimates that they are capable of attaining 42 to 46 population doublings.

1.4 Add the amount of cell suspension required to each bottle and place all bottles on a roller rack at 37° C in a humidified 5% $CO_2$ incubator.

1.5 Repeat this procedure with 2 more cell factories each time, until the appropriate number of roller bottles have been seeded.

**2.0 INFECTION OF CELLS (2 DAYS POST-SEEDING)**

2.1 Microscopically examine the roller bottles to ensure that the cells are healthy and about 50% confluent.

2.2 Prepare the infection medium need for each group of 20 roller bottles (Falcon, expanded surface 1450 $cm^2$ surface area) to be infected.

2.2.1 The infection medium for each group of 20 roller bottles contains 1260mL of EMEM + 10% FBS. Each roller bottle is infected with 60mL infection medium and this allows an excess of 1 roller bottle.

2.2.2 Calculate amount of virus needed for infection of each group at an MOI of 50 particles/cell.

2.2.2.1 The average number of cells per roller bottle has been determined to be $4x10^7$ cells on the day of infection.

2.2.2.2 To obtain an MOI of 50, each roller bottle will be infected with $2x10^9$ virus particles.

2.2.2.3 For each group of 20 roller bottles, $4.2x10^{10}$ virus particles are required for each 1260mL of infection medium.

2.2.3 Thaw virus quickly to room temperature and add the appropriate amount virus to the infection medium just before use.

2.3 Remove the roller bottles from the incubator one group at a time, label each bottle (with the group identification and number of times the virus has been passaged on MRC-5 cells).

2.4 Aspirate the medium from each roller bottle and add 60mL of infection medium (with virus added) to each roller bottle.

2.5 Place roller bottles back on a roller rack at 37°C in a humidified 5% $CO_2$ incubator.

2.6 Clean hood and start infection of the next group of roller bottles (steps 2.2 through 2.5).

2.7 After the roller bottles have been incubated for at least 4 hours on a roller rack, add EMEM Recovery Medium (EMEM + 10% FBS + 1% Pen-Strep + 1% Fungizone) to each group in the same order as infection, cleaning hood between each group.

2.7.1 If there is a partial bottle of medium remaining after all roller bottles from the group are completed, discard the medium and do not use it for the next group.

2.8 Incubate infected cells on a roller rack at 37°C in a humidified 5% $CO_2$ incubator.

## 3.0 REFEEDING INFECTED ROLLER BOTTLES

3.1 Refeed all groups of roller bottles in the same order as infected.

3.1.1 Refeeding is performed two days after infection, if infection occurs on Wednesday. This means that roller bottles were seeded on Monday, infected on Wednesday, and refed on Friday.

3.1.2 Refeeding is performed three days after infection, if infection occurs on Friday. This means that roller bottles were seeded on Wednesday, infected on Friday, and refed the following Monday.

3.2 Aspirate medium from one group of roller bottles and add 300mL EMEM Recovery Medium to each bottle. Do not use the same bottle of medium for more than one group of roller bottles.

3.3 Repeat the process until all groups have been refed, cleaning the hood between groups.

3.4 Incubate infected cells on a roller rack at 37°C in a humidified 5% $CO_2$ incubator.

## 4.0 CELL HARVEST (6 DAYS POST-INFECTION)

4.1 Observe the infected roller bottles under the microscope on the day of harvest to see how much CPE is present.

**NOTE:** As passage on MRC-5 cells increases, the CPE might also increase, if a more potent recombinant is present. It is possible that by the fourth passage on MRC-5 cells, the cells will be showing so much CPE that they will come off of the roller bottles as in a normal prep. These roller bottles are harvested without removal of the medium and addition of Versene. They are harvested by shaking the roller bottles to remove cells and centrifuging the whole suspension. Day of harvest may be decreased to 5 days post-infection for those samples in which CPE is present earlier.

4.2 Harvest roller bottles, one group at a time, in the order of infection.

4.2.1 Repeat the entire harvesting process below (steps 4.3 through 4.14) for each group, cleaning the hood(s) between groups.

4.2.2 Two groups can be harvested at the same time, in an overlapping fashion, by using a separate hood for each group.

4.3 Aspirate medium from each roller bottle in the group and add 50ml Versene to each roller bottle. Do not use the same bottle of Versene for more than one group of roller bottles.

4.4 Place roller bottles back on the roller rack and incubate for approximately 20 min. at 37°C in a humidified 5% $CO_2$ incubator.

**NOTE:** The time required for incubation with Versene will depend on the level of CPE.

4.5 Make sure caps are closed tightly and bang the roller bottles very sharply. Try to get all cells off of the surface.

4.6 Pipette the Versene from each roller bottle into 500ml centrifuge tubes. Add contents of 5 roller bottles to each 500ml tube. This means you will use 4 tubes for each group of 20 roller bottles.

4.7 Slowly add 50ml EMEM Recovery Medium to each roller bottle, rotating the bottle as you drip the medium down the sides to rinse. Add this medium to the same tube containing the Versene suspension

from these roller bottles. Do not use the same bottle of medium for more than one group.

4.8 Centrifuge tubes at 2000 rpm for 5 minutes at room temperature with no brake.

4.9 Pour off or aspirate supernatant and combine cell pellets into one 500mL tube with 400 to 450mL DPBS. Reserve about 50 to 100mL DPBS for the final resuspension of the pellet. Do not use the same bottle of DPBS for more than one group.

4.10 Centrifuge the 500mL tube again at 2000 rpm for 5 minutes at room temperature with no brake.

4.11 Aspirate supernatant and estimate the volume of the pellet. Resuspend the pellet in 5mL of DPBS. Use a sterile 10mL pipette to measure the suspension.

4.12 Pipette the cell suspension up and down to make a slurry. Transfer the slurry into 50mL conical tubes as 10mL aliquots. The tubes are labeled with group identification and tube # of total #.

4.13 Determine the volume of DPBS needed to bring the final volume of cell suspension to 2.5 times the initial volume of cell pellet, rinse the 500mL tube with at least this volume of DPBS, and add this volume evenly to the tubes of cell suspension.

4.14 Store tubes at -65° C to -85°C until purification is initiated.

## 5.0 PURIFICATION PROCEDURE

5.1 Freeze/Thaws

5.1.1 Using dry ice and a 37°C water-bath, perform a series of 3 freeze/thaw cycles on the cell suspension. The thaw time will vary due to the differing volume of CVL in each tube. Check the tubes in the thaw cycle periodically since excess time at 37°C may result in lower virus viability.

5.1.2 Vortex tubes for at least 10 seconds after each thaw. The vortexing is required to help release the virus into the DPBS. NOTE: The CVL may be stored at –65 to –85°C during any of the freeze cycles.

5.1.3 One final thaw is performed immediately before beginning the discontinuous gradient.

5.2 Discontinuous Gradient

5.2.1 After the final thaw, centrifuge the tubes at 3000 rpm with the brake off for 5 minutes at room temperature, to clear the final freeze/thaw suspension.

5.2.2 Remove the cleared CVL with a pipette from the centrifuge tube. Place cleared CVL in sterile 50mL tube(s).

NOTE: If there is a question as to whether or not you have produced enough virus to purify, the cleared CVL can be tested by HPLC to determine particle titer. The CVL should be kept frozen at –80°C while waiting for results. Once particle titer is available, the decision can be made to purify (need to start with approximately 1.5 to $2x10^{12}$ virus particles) or to infect the next passage using CVL.

5.2.3 Divide the total volume of the CVL by seven to get the number of gradients. For each gradient to be run, remove 3.5mL aliquots each of CsCl (1.25 g/mL) and CsCl (1.40 g/mL) to sterile 50mL tubes in the clean hood. Do not open stock bottles in the virus hood. Remove aliquots for preparing a balance tube if one will be required.

5.2.4 Set up one Beckman SW28.1 ultraclear tube for each gradient in the virus hood, and place a sterile Pasteur pipet into the neck of each tube.

5.2.5 Add 3mL of CsCl (1.25 g/mL) through the Pasteur pipet for each tube. Slowly underlay 3mL of CsCl (1.40 g/mL) through the Pasteur pipet. Carefully remove the pipette and place in the sharp container.

NOTE: Check to see that the gradient interface has formed. This appears as a silver or gray line in the middle of the liquid. If the line hasn't formed, dispose of the cesium and set up a new tube.

5.2.6 Attach an 18 gauge, 1.5-inch needle to a 10cc syringe with the plunger removed. Insert the needle into the neck of the tube in place of the Pasteur pipet.

5.2.7 Add 5-7 mL of CVL into the syringe. Do not load more than 7mL of CVL on a single gradient. To avoid disrupting the gradient, allow the CVL to drip from the needle. Dispose of the needle in the sharp container.

5.2.8 With a clean syringe/needle add DPBS into each tube, completely filling each tube excluding the neck.

5.2.9 In the virus hood, weigh the tubes and balance pairs within 0.05g, using DPBS to balance the tubes.

5.2.10 Using the Beckman heat sealer, heat-seal the tubes. Place the metal caps on top of each tube. Pressing the red button, touch the sealer to the metal cap. Allow the sealer to melt the tube until the metal cap reaches the rounded part of the tube. After the cap reaches the top of the tube, hold the metal cap in place with the black rod and allow the plastic to cool. Once the plastic has set pull the metal cap off. Rinse metal caps with anti-viral disinfectant and alcohol.

5.2.11 Place the tubes into the SW 28.1 buckets. Then add the SW 28.1 black spacers to the top of the tube.

5.2.12 Match the lid of the bucket to the number on the bucket. Screw the lid on until it is tight and the numbers on the lid and bucket match up. The balanced pairs must be run as a pair. Bucket 1 and 4, 2 and 5, and 3 and 6, are the pairs.

5.2.13 Load buckets onto SW 28 swinging bucket rotor. This also requires the numbers on the rotor and the bucket to match up. Give the bucket a slight tug to ensure that the bucket is securely attached to the rotor. All six buckets must be run. Run buckets empty if fewer than 6 tubes are set up.

5.2.14 Turn on the Beckman Ultracentrifuge. Place the rotor in the centrifuge and close the door. Go to program library. Find the program number that corresponds to 1 hour at 28,000 rpm at 20°C. Press the number and enter. Then press the rotor

button and pick the correct serial number for the rotor being run and press enter. Press enter one more time (total of twice) and press the start button.

5.2.15 After centrifugation, press the vacuum button on the centrifuge. Remove the rotor. Open the lid and pull out the spacers and then the tube using hemostats. Return to the virus hood. Two bands should be visible. The bottom band is the purified virus. The upper band is cellular debris. A dark or black background behind the tube may make it easier to clearly see the bands. NOTE: If tube is stuck in the bucket, grasp tube with hemostats and twist the tube while lifting. Be careful not to jiggle the tube too much. If the tube is leaking because of collapsing, return the rotor/bucket to the virus hood and remove the tube.

5.2.16 Wipe or spray the tube with alcohol and allow to dry. Puncture the top of the tube with a 1-inch needle to break the vacuum.

5.2.17 Using an 18 gauge, 1.5-inch needle attached to a syringe, carefully puncture the tube about 1cm below the bottom band. Slowly pull back on the plunger of the syringe to collect the band, being sure to harvest only the bottom band. Remove the needle and place the virus in a sterile 50mL tube. The tube from which you collect the virus with cesium in it is discarded in the sharp container. Discard all needles as sharp waste.

5.3 Continuous Gradient (Overnight)

5.3.1 The total volume of virus that has been collected from the discontinuous gradients in a sterile 50mL tube should be divided by seven for the number of gradients that will be set up for the overnight gradient.

5.3.2 Set up one NVT polyallomer tube for each gradient in the virus hood, and place a sterile Pasteur pipet into the neck of each tube. Add the virus with a pipette through the Pasteur pipet. Do not load more than 7mL of virus per tube.

5.3.3 In the clean hood, remove enough CsCl (1.33 g/mL) to fill each tube completely with CsCl. The total capacity of the tube is

approximately 13mL. Remember to remove aliquots for preparing a balance tube if one is needed.

5.3.4 After filling the NVT tube with virus, fill each tube to be centrifuged completely with CsCl (1.33 g/mL), using a syringe/needle. If running a blank, fill the tube completely with CsCl (1.33 g/mL).

5.3.5 Balance pairs of tubes within 0.05g using CsCl (1.33 g/mL) to balance and heat seal as before.

5.3.6 Place tubes in the NVT rotor and place the blue inserts on the tops of the tubes. Place the rotor lids on. Using the torque wrench, tighten the rotor lids to 120 inch pounds. Empty chambers should be run without caps or inserts.

5.3.7 Place the rotor in the centrifuge and close the door. Go to program library. Find the program number that corresponds to "hold" at 60,000 rpm at 20°C. Press the number and enter. Then press the rotor button and pick the correct serial number for the rotor being run and press enter. Press enter one more time (total of twice) and press the start button. Record information needed in the Beckman Log Book.

5.3.8 The overnight run must be a minimum of 15 hours. Press the stop button. After the spin is complete, press the vacuum button. Take the rotor from the centrifuge and pull the bottom bands as before.

5.4 Dialysis

5.4.1 Add glycerin to the virus to make a final concentration that is approximately 10% glycerin.

5.4.2 Thoroughly mix the virus and the glycerin by pipetting up and down in the tube.

5.4.3 Remove up to 3.5mL of virus with a syringe and an 18 gauge, 1-inch needle. Inject the virus into the Slide-A-Lyzer dialysis cassette (10,000 MW cut-off, Pierce Cat #66425) through port #1.

5.4.4 Before removing the needle from the cassette, pull back on the plunger of the syringe to remove the air and utilize all of the

surface area of the cassette. It may be necessary to go to another port to remove all of the air. Use each port only once when injecting virus because ports may leak if used more than once.

5.4.5 Each cassette is dialyzed in one liter of cold THPG dialysis buffer (200mM Tris, 50 mM Hepes, 0.3% o-Phosphoric acid, 10% Glycerin).

5.4.5.1 Place the cassettes in an autoclaved foil-covered beaker with a stir bar, one group per beaker.

5.4.5.2 Beakers are placed at 2-8°C on a stir plate set on medium-low speed.

5.4.5.3 There will be a total of three changes of dialysis buffer. There is at least one hour between changes. For the changes, aspirate the buffer from the beaker, then add the same amount of buffer as before. One change continues overnight, for a minimum of 10 hours.

## 6.0 PARTICLE TITER DETERMINATION AND FILL

6.1.1 Turn on the UV lamp on the spectrophotometer prior to use. Turn on the visible light lamp immediately before use.

6.1.2 Take syringe with needle and pull the plunger back to capture 1 cc of air. Insert the needle into the cassette port. Slowly push down on the plunger to inject air into the dialysis cassette.

6.1.3 Flip the cassette upside down and pull back on the plunger to remove the virus from the cassette. Remove the needle from the syringe using hemostats, place the virus in a sterile 50mL tube.

6.1.4 Keep the virus in the refrigerator until aliquoted.

6.1.5 Determine the particle titer by optical density ($OD_{260}$).

6.1.5.1 Mix 3.75mL of TE buffer and 40µl of 10% SDS in a 15mL tube, to serve as Lysis buffer.

6.1.5.2 Label 2 non-sterile Eppendorf tubes per virus sample and one for a blank.

NOTE: It may be necessary to use a lower dilution (such as 1:5) if the virus is suspected to have a lower particle titer.

| | Volume of Lysis buffer | Volume of virus |
|---|---|---|
| Sample-1:10 dilution (10x) | 360µL | 40µL |
| Sample-1:20 dilution (20x) | 380µL | 20µL |
| Blank | 400µL | None |

6.1.5.3 Using a 1000µL pipetter add the correct amount of Lysis buffer to the corresponding tubes from the chart above. Using a 20µL or 200µL pipetter add the amount of virus indicated in the chart to the 20x and 10x tubes. Be sure to use a new tip each time to ensure that Lysis buffer does not get into the virus and to keep the virus sterile.

6.1.5.4 Vortex the tubes for approximately 10 seconds at a setting of 5.

6.1.5.5 Allow the solutions to sit at RT for 15-30 minutes before taking the readings.

6.1.5.6 Rinse the cuvette thoroughly with rinse water before adding the blank. Use all 400µL of the blank when reading. Read the blank as a blank at a wavelength of 260nm and then as a sample on the spectrophotometer.

6.1.5.7 Rinse the cuvette thoroughly with rinse water before adding the samples. Read the 20x dilution before the 10x dilution. Rinse the cuvette with rinse water in between each reading.

6.1.5.8 After the results appear on the screen print out the data using the "print" command. Use the Save/Clear command to allow the machine to exit. Write on the printout indicating which line is the blank, 10x dilution, and 20x dilution of each sample. Initial and date the printout.

6.1.5.9 Determine the concentration of the virus by multiplying the $OD_{260}$ by the dilution (10 or 20) and conversion factor ($1.1x10^{12}$). Final results should be rounded to three significant figures.

6.2 Aliquot the virus.

6.2.1 Label the cryovials with the following:

Sample Number
Name of virus and Passage # on MRC-5
# of particles/mL
Date aliquoted Volume of aliquot
Store at -80C

6.2.2 Virus aliquots are stored in a -80°C freezer.

## 7.0 FURTHER PASSAGES OF VIRUS ON MRC-5 CELLS

7.1 Repeat the full procedure three more times using the purified virus or CVL produced from the passage before.

7.2 Once four passages have been completed, perform endpoint analyses and data interpretation as described.

## 8.0 ENDPOINT ANALYSES

8.1 Determine virus production at the end of each passage

8.2 Assess cytopathic effect (CPE) at the end of each passage

8.3 If there is evidence of increased virus yield and CPE, the following additional analyses may be performed on virus harvested from any passage:

8.3.1 Perform restriction digests on harvested virus.

8.3.2 Determine potency of harvested virus on MRC-5 cells using a cytotoxicity assay with MTS readout.

8.3.3 Assess replication competence by hexon FACS analyses following infection of indicator cells

## 9.0 DATA INTERPRETATION

9.1 CPE and increased production at the end of four passages is interpreted as the presence of a replication competent adenovirus (RCA)

9.2 Aberrant banding patterns on restriction digest gels after passage on MRC5 cells also is interpreted as the presence of recombinant adenovirus.

9.3 Increased potency after passage on MRC5 cells is interpreted as a recombinant that has lost selectivity.

9.4

**EXAMPLE 14: Cytotoxicity potency assay with MTS readout.**

MRC-5 cells are seeded in 96-well tissue culture dishes at 10,000 cells per well in 100 µl 10% EMEM. The next day, adenoviruses are serially diluted 1:3 in 10% EMEM. The growth medium from each plate is removed and 100 µl of each viral dilution are added to the appropriate wells on each of four plates. The dilution ranges tested are $3.3 \times 10^8$ to $7.0 \times 10^1$ vp/mL (or $3.3 \times 10^3$ to $7.0 \times 10^{-4}$ particles per cell, ppc). Cells are exposed to virus for ten days after which the Promega CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS assay) is performed according to the manufacturer's instructions (Promega Tech. Bulletin #TB169). Absorbance units are converted to percent uninfected control values (% Max Control) and replicates are averaged (± SD) between the four plates. A sigmoidal dose-response curve of average % Max Control plotted versus vector viral particle dose (ppc) in logarithmic scale is fit to the data and an EC50 value is determined for each virus tested, using GraphPad Prism software.

**EXAMPLE 15: Determination of viral vector particle concentration by OD260/SDS procedure.**

Samples are diluted at 1:10 and 1:20 into lysis buffer (0.106% SDS in TE buffer; BioWhittaker #16-013B) and incubated for 15-30 min at room temperature prior to determining absorbance readings. Samples are analyzed using a Beckman DU 640 spectrophotometer. The absorbance reading using lysis buffer only is adjusted to zero at 260 nm, and then absorbance readings of each dilution are obtained. Viral particle concentrations are determined using the following formula (Maizel, et al 1968): Concentration (particles/ml) = (A260 nm) x ($1.1 \times 10^{12}$ particles/ml) x (1/dilution factor)

A similar procedure is described by Mittereder et al. (J Virol.1996 Nov ;70(11): 7498-509)

**EXAMPLE 16: Quantitative PCR.**

All PCR reactions are performed in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). PCR data is analyzed using the Sequence Detection System software version 1.6.3 (Applied Biosystems).

To quantify the amount of wild type Ad5 in purified oncolytic virus samples, a quantitative real-time PCR is designed to detect the wild-type E1A promoter. A standard curve is generated using 1.2 μg (3 x $10^{10}$ viral genomes) of Ar6pAE2fE3F viral genomic DNA as background mixed with known amounts of Ad5 viral genomic DNA, ranging from 10 to 1 x $10^{6}$ viral genomes. A negative control of 1.3 μg (3 x $10^{10}$ plasmid copies) of plasmid containing the Ar6pAE2fE3F sequence is included in each assay. Each 100 μl reaction of standard or unknown contains: 1.2 μg DNA (3 x $10^{10}$ viral genomes), 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 μM of each primer (forward primer 5'-GACCGTTTACGTGGAGACTCG-3' (SEQ ID NO:1) and reverse primer 5'-TCGGAGCGGCTCGGA-3' (SEQ ID NO:2)) and 0.1 μM of the probe (5'-FAM-TTTTCCGCGTTCCGGGTCAAAGT-TAMRA-3' ((SEQ ID NO:3)). The PCR reactions are performed under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 60 cycles of 95°C for 15 sec and 60°C for 1.5 min.

Adenoviruses can be grown (amplified) on PerC.6 cells. PerC.6 cells contain an expression cassette with Ad5 E1a operably linked to a PGK promoter. If adenoviruses are grown on a PerC.6 cell line there is a potential for the PGK promoter to be incorporated in to the adenovirus. The PGK promoter has been reported to incorporate into the adenovirus gemone between the ITR and the E1 region in such a way that it is operably linked to the adenovirus E1 coding regions. Thus replacing or interfering with any promoter that may have been operably linked to the E1 coding region to selectively control replication of the virus.

To determine the amount of PGK promoter-containing recombinants in adenoviral preparations, (if any) the PCR assay is designed to detect adenoviral sequences containing the PGK promoter/E1A junction as found in PER.C6 cells. A standard curve is generated using a background of 5 x $10^{10}$ PGK promoter-negative adenoviral genomes (2 ug of adenoviral genomic DNA) mixed with 50 to 1 x $10^{6}$ copies of a plasmid containing the PGK/E1a junction sequence. Each 100 μl reaction of either standard or unknown sample contains: 2 ug adenoviral genomic DNA, 1x TaqMan Universal PCR Master Mix, 0.3 μM of each primer (forward primer 5'-

CCGCACGTCTCACTAGTACCC-3' (SEQ ID NO:4) and reverse primer 5'-ACACGATCGAATTCGGAACG-3' (SEQ ID NO:5)) and 0.1 μM of the probe (probe 5'-6FAM-CGGAGCGGGATCGAGCCCTCT-TAMRA-3' (SEQ ID NO:6)). The PCR reactions are carried out under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 60 cycles of 95°C for 15 sec and 60°C for 1.5 min. In one of these reactions the limit of detection of the assay was 50 copies of PGK/E1a per 5 x $10^{10}$ adenoviral genomes.

To quantitate REC133, a standard curve is generated using 10 to 1 x $10^6$ copies of a plasmid containing a cloned REC133 restriction fragment in a background of 1 x $10^8$ adenoviral genomes (4 ng adenovioral genomic DNA). Each 100 μl PCR reaction contains: 4 ng adenoviral genomic DNA, 1x TaqMan Universal PCR Master Mix, 1 μM of each primer (forward primer 5'-TTTCTGGGCGTAGGTTCGC-3' (SEQ ID NO:7) and reverse primer 5'-GGTAATAACACCTCCGTGGCA-3' (SEQ ID NO:8)) and 0.2 μM of the probe (probe 5'-6FAM-CGGAGCGGGATCGAGCCCTCT-TAMRA-3' (SEQ ID NO:9)). The PCR reactions are carried out under the following conditions: 50°C for 2 min, 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 64°C for 1 min. In one of these reactions tbe limit of detection of the assay was 10 copies of REC133 per 1 x $10^8$ adenoviral genomes.

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The disclosures of all patents, patent applications, publications (including published patent applications), and database accession numbers referred to in this specification are specifically incorporated herein by reference in their entirety to the same extent as if each such individual patent, patent application, publication, and database number were specifically and individually indicated to be incorporated by reference in its entirety.

1. A method for detecting a non-selective replication competent virus (RCV) in a preparation of selectively-replicating virus, comprising:

(a) passaging the virus preparation at least once on cells that differentially amplify the selectively-replicating virus versus a replication competent virus; and

(b) analyzing the virus preparation amplified in (a), thereby detecting the presence of a replication competent virus, wherein analyzing the virus preparation comprises one or more of the following: detecting increased cytopathic effect; detecting increased virus production; detecting increased potency to kill normal cells; detecting an altered restriction digest pattern; detecting an altered viral genome sequence; detecting anticipated recombinants with PCR amplification; and detecting acute hepatotoxicity in an animal administered the virus preparation.

2. The method according to claim 1, wherein 10-100 copies of the non-selective replication competent virus can be detected in $1 \times 10^8$ selectively-replicating virus particles.

3. The method according to claim 1, wherein the virus preparation is passaged in (a) on MRC5 cells.

4. The method according to claim 1, wherein increased virus production is measured in normal human cells with a hexon FACS assay.

5. The method according to claim 1, wherein increased potency to kill normal cells is measured with an MTS assay.

6. The method according to claim 1, wherein acute hepatotoxicity is detected in a SCID mouse administered the virus preparation.

7. The method according to claim 1, further comprising the step of selecting cells for their ability to differentially amplify the selectively-replicating virus versus a replication competent virus, prior to passaging and analyzing the virus preparation.

8. The method according to claim 1, wherein the virus preparation is comprised of a virus selected from the group consisting of adenovirus, herpesvirus, reovirus, paramyxovirus, sindbis, parvoviruses, poxvirus, picornavirus, orthonyxoviruses, and rhabdovirus.

9. The method according to claim 8, wherein the virus preparation is an adenovirus preparation.

10. The method according to claim 9, wherein the adenovirus preparation is comprised of a tissue-specific replication-conditional adenovirus.

11. The method according to claim 10, wherein the tissue-specific replication-conditional adenovirus is tumor-specific.

12. The method according to claim 11, wherein the tumor-specific replication-conditional adenovirus comprises a mutation or deletion in the E1b gene, wherein the encoded E1b protein lacks the capacity to bind p53.

13. The method according to claim 11, wherein the tumor-specific replication-conditional adenovirus comprises a mutation or deletion in the E1a gene, wherein the encoded E1a protein lacks the capacity to bind RB.

14. The method according to claim 10, wherein the replication-conditional adenovirus comprises a mutation or deletion in the E3 region.

15. The method according to claim 11, wherein the tissue-specific replication-conditional adenovirus comprises a heterologous tissue-specific transcriptional regulatory sequence operatively linked to the coding region of a gene that is essential for replication of the adenovirus, wherein the transcriptional regulatory sequence functions in the tissue so that replication of the virus occurs in the tissue.

16. The method according to claim 15, wherein the transcriptional regulatory sequence is a promoter or an enhancer.

17. The method according to claim 16, wherein the transcriptional regulatory sequence is selected from the group consisting of an E2F-responsive promoter, a human telomerase reverse transcriptase (hTERT) promoter, an osteocalcin promoter, a carcinoembryonic antigen (CEA) promoter, a DF3 promoter, an α-fetoprotein promoter, an ErbB2 promoter, a surfactant promoter, a tyrosinase promoter, a MUC1/DF3 promoter, a TK promoter, a p21 promoter, a cyclin promoter, an HKLK2 promoter, a uPA promoter, a HER-2neu promoter, a prostate specific antigen (PSA) promoter, a probasin promoter, a human uroplakin II-derived transcriptional regulatory element, and a melanoma cell specific transcriptional regulatory element (TRE).

18. The method according to claim 17, wherein transcriptional regulatory sequence is an E2F-1 promoter.

19. The method according to claim 15, wherein the adenovirus coding region that is operatively linked to the transcriptional regulatory sequence is an E1, E2, or E4 coding region.

20. The method according to claim 19, wherein the E1 coding region is an E1a or E1b coding region.

21. The method according to claim 19, wherein the E2 coding region is an E2a or E2b coding region.

22. The method according to claim 15, wherein the adenovirus comprises an E2F-1 promoter operatively linked to the E1a coding region.

23. The method according to claim 15, wherein the virus further comprises a second heterologous tissue-specific transcriptional regulatory sequence operatively linked to the coding region of a second gene that is essential for replication of the virus, wherein the second transcriptional regulatory sequence functions in the tissue so that replication of the virus occurs in the tissue.

24. The method according to claim 23, wherein the adenovirus comprises an E2F-1 promoter operatively linked to the E1a coding region and an hTERT promoter operatively linked to the E4 coding region.

25. The method according to claim 23, wherein the adenovirus comprises a probasin promoter operatively linked to the E1a coding region and a prostate specific antigen promoter/enhancer operatively linked to the E1b coding region.

26. The method according to claim 1, wherein the virus further comprises a heterologous gene coding sequence.

27. The method according to claim 26, wherein the heterologous gene coding sequence provides anti-tumor activity in the cells of a target tissue.

28. The method according to claim 26, wherein the heterologous gene coding sequence encodes GM-CSF.

29. The method according to claim 9, wherein the adenovirus is derived from a human adenovirus 5 genome or a human adenovirus 35 genome.

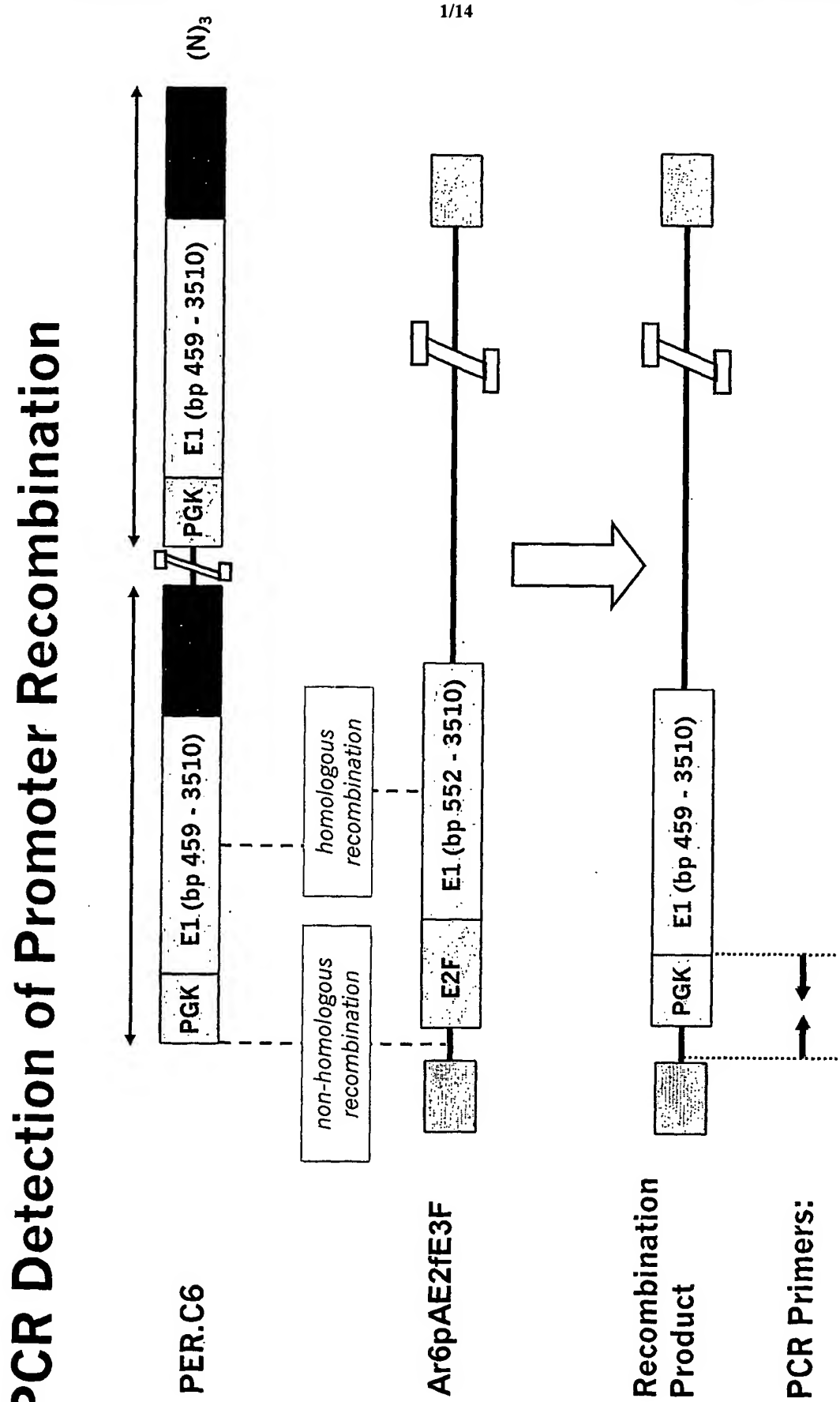
PCR Detection of Promoter Recombination
PER.C6
PGK
E1 (bp 459 - 3510)
(N)3
non-homologous recombination
homologous recombination
Ar6pAE2fE3F
E2F
E1 (bp 552 - 3510)
Recombination Product
PGK
E1 (bp 459 - 3510)
PCR Primers:

Figure 1

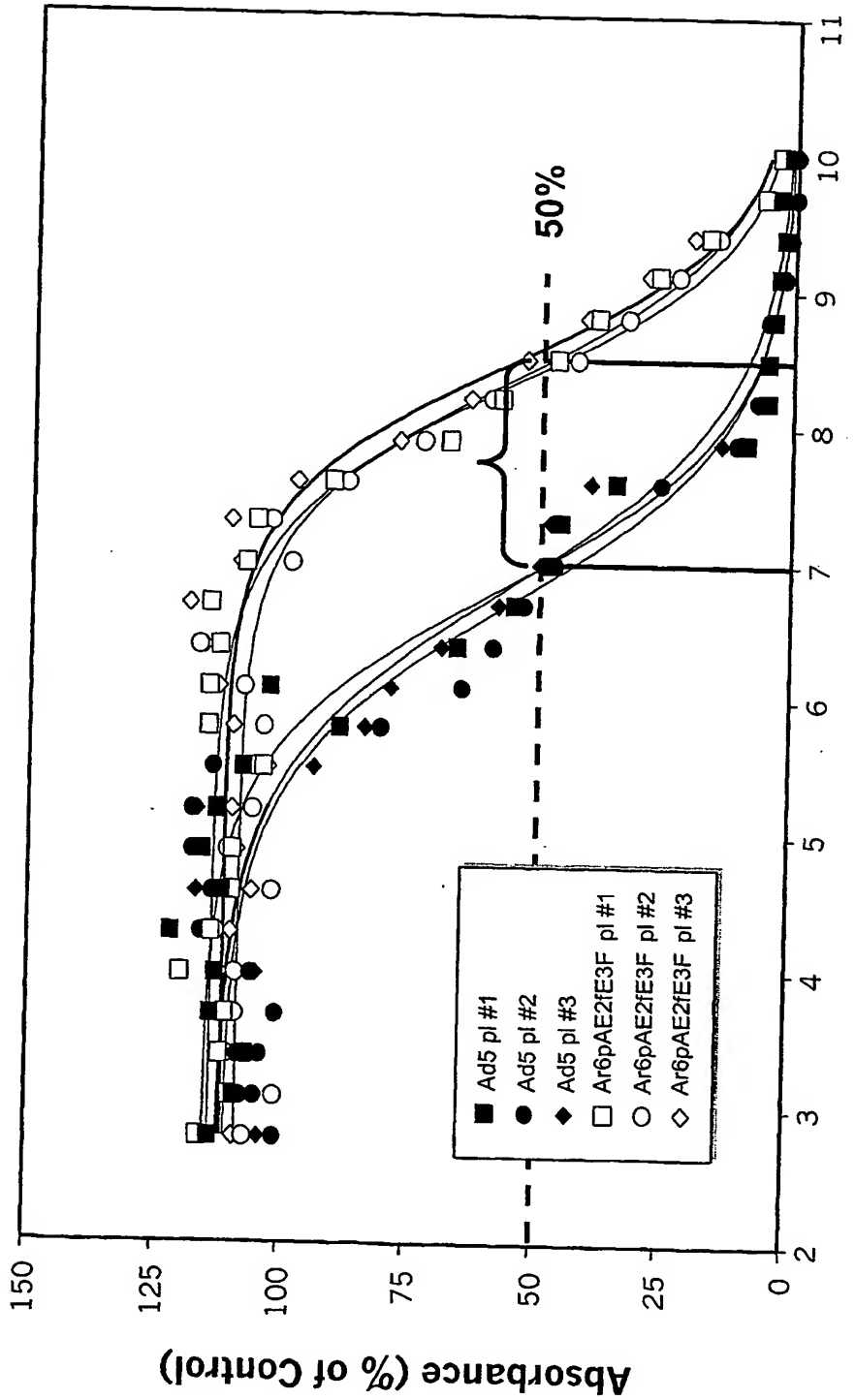
Absorbance (% of Control)
150
125
100
75
50
25
0
2
3
4
5
6
7
8
9
10
11
50%
Ad5 pl #1
Ad5 pl #2
Ad5 pl #3
Ar6pAE2fE3F pl #1
Ar6pAE2fE3F pl #2
Ar6pAE2fE3F pl #3
[Virus, log10] (vp/mL)

Figure 2

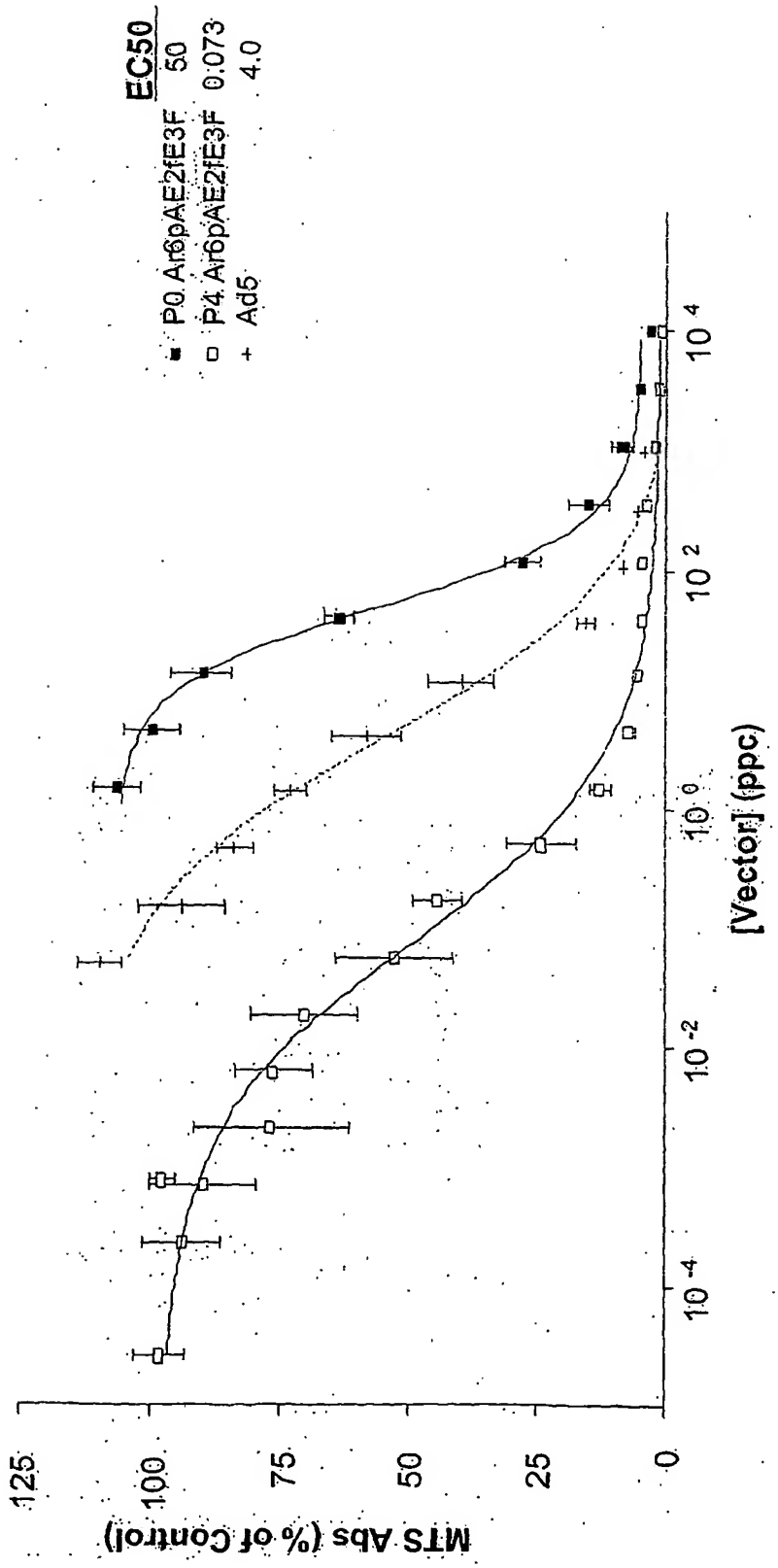
EC50
P0 Ar6pAE2fE3F 50
P4 Ar6pAE2fE3F 0.073
Ad5 4.0
MTS Abs (% of Control)
125
100
75
50
25
0
[Vector] (ppc)
10 -4
10 -2
10 0
10 2
10 4

Figure 3

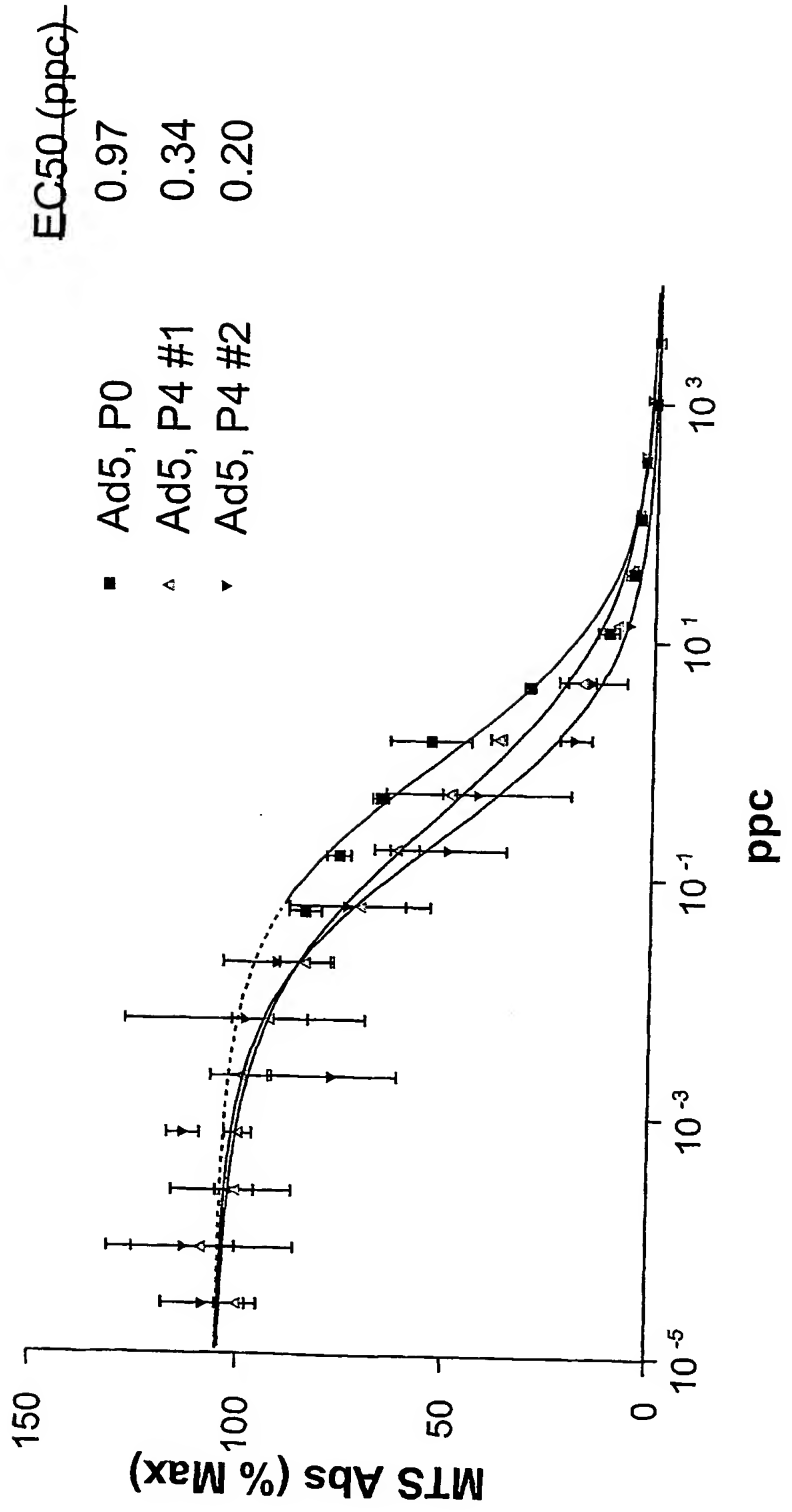
EC50 (ppc)
Ad5, P0 0.97
Ad5, P4 #1 0.34
Ad5, P4 #2 0.20
MTS Abs (% Max)
150
100
50
0
10 -5
10 -3
10 -1
10 1
10 3
ppc

Figure 4

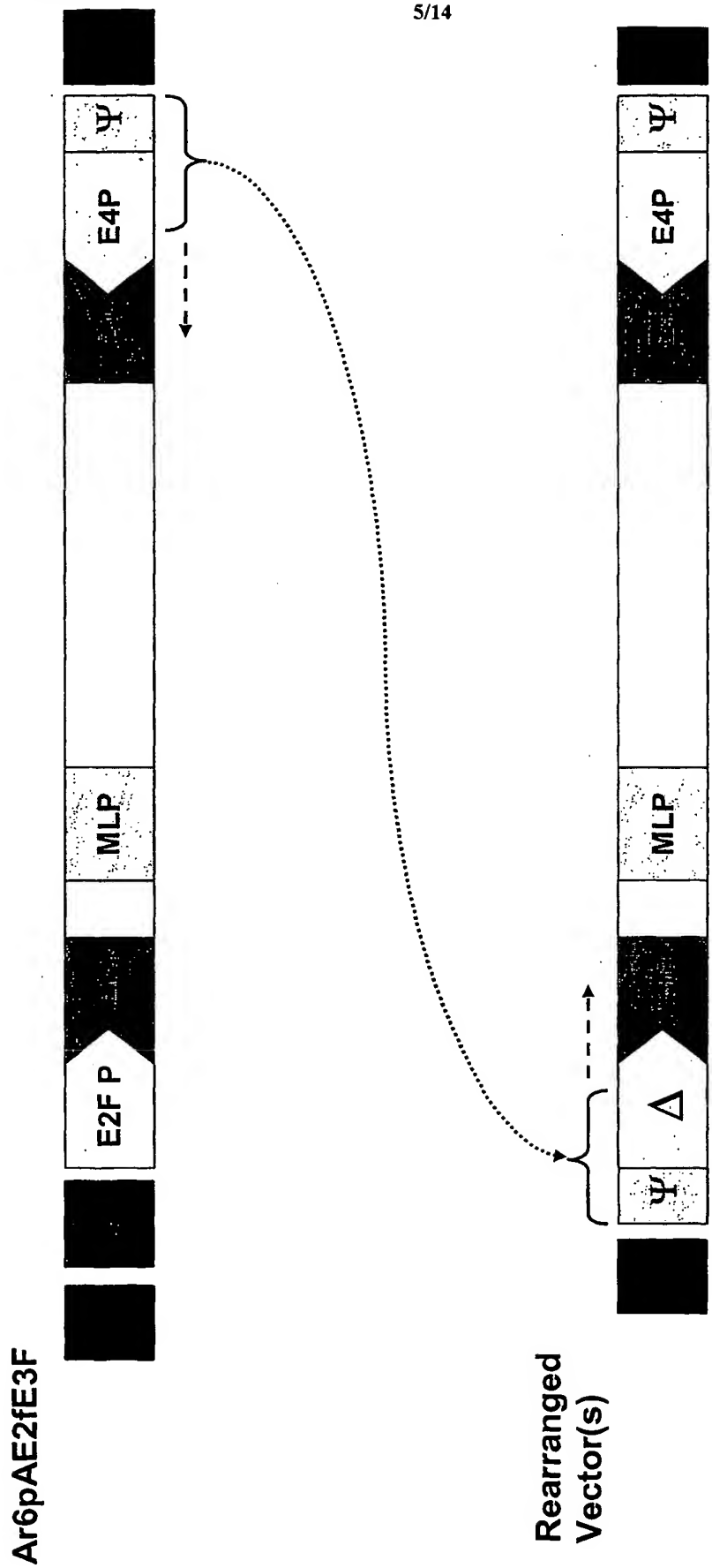


Figure 5

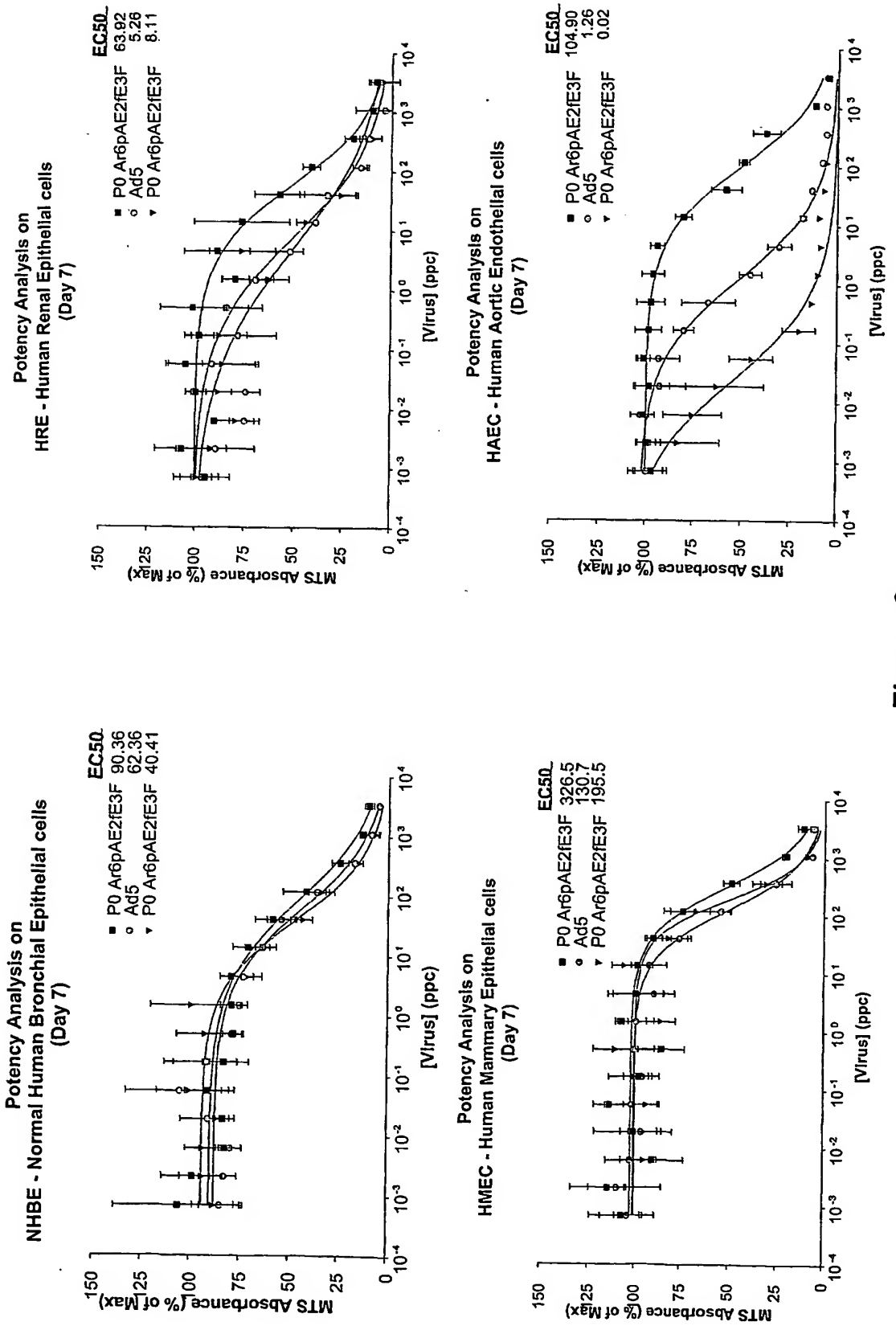
Potency Analysis on
HRE - Human Renal Epithelial cells
(Day 7)
EC50
P0 Ar6pAE2fE3F 63.92
Ad5 5.26
P0 Ar6pAE2fE3F 8.11
MTS Absorbance (% of Max)
[Virus] (ppc)
Potency Analysis on
HAEC - Human Aortic Endothelial cells
(Day 7)
EC50
P0 Ar6pAE2fE3F 104.90
Ad5 1.26
P0 Ar6pAE2fE3F 0.02
MTS Absorbance (% of Max)
[Virus] (ppc)
Potency Analysis on
NHBE - Normal Human Bronchial Epithelial cells
(Day 7)
EC50
P0 Ar6pAE2fE3F 90.36
Ad5 62.36
P0 Ar6pAE2fE3F 40.41
MTS Absorbance (% of Max)
[Virus] (ppc)
Potency Analysis on
HMEC - Human Mammary Epithelial cells
(Day 7)
EC50
P0 Ar6pAE2fE3F 326.5
Ad5 130.7
P0 Ar6pAE2fE3F 195.5
MTS Absorbance (% of Max)
[Virus] (ppc)

Figure 6

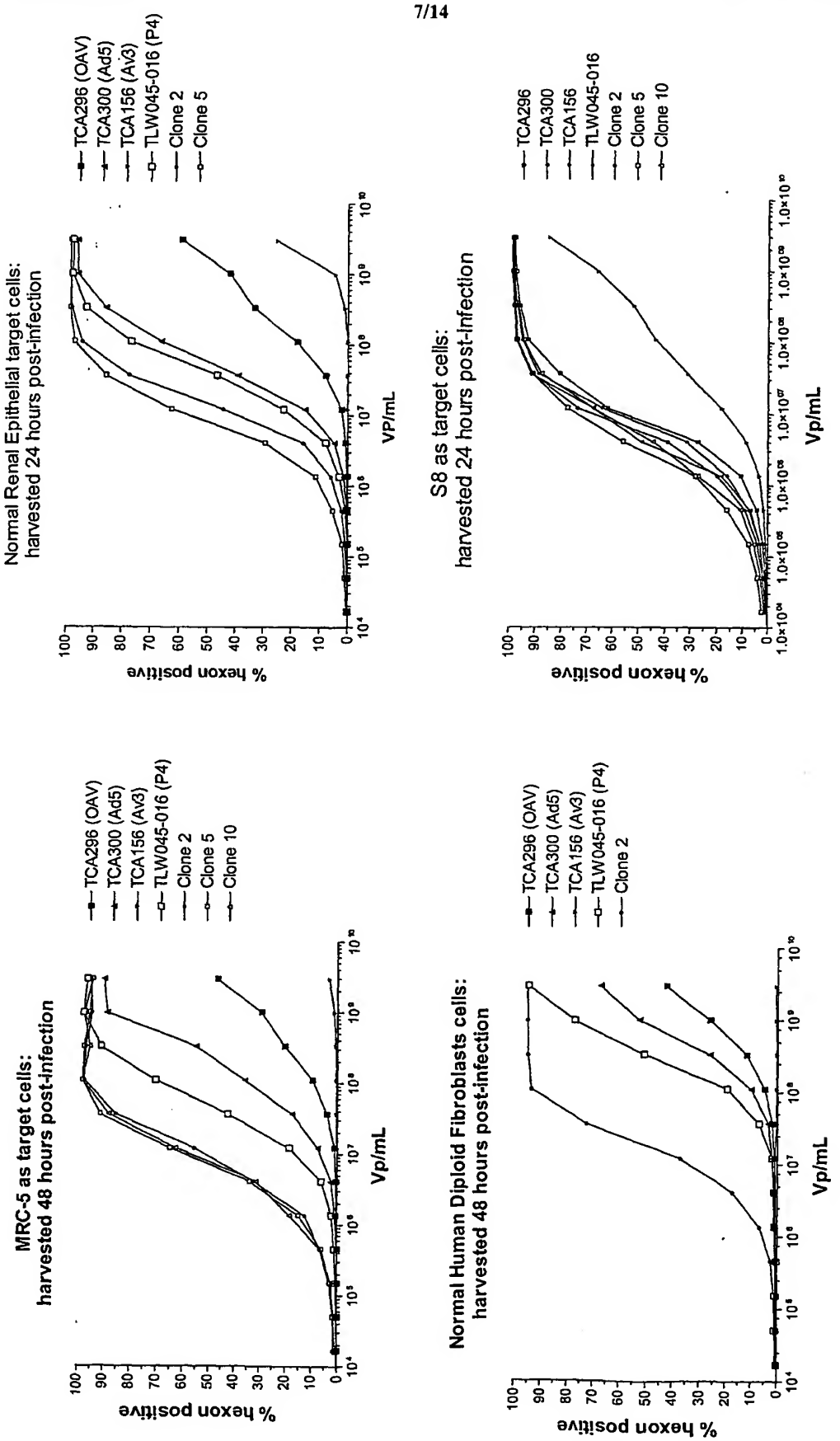
Normal Renal Epithelial target cells:
harvested 24 hours post-infection
TCA296 (OAV)
TCA300 (Ad5)
TCA156 (Av3)
TLW045-016 (P4)
Clone 2
Clone 5
% hexon positive
VP/mL
S8 as target cells:
harvested 24 hours post-infection
TCA296
TCA300
TCA156
TLW045-016
Clone 2
Clone 5
Clone 10
% hexon positive
Vp/mL
MRC-5 as target cells:
harvested 48 hours post-infection
TCA296 (OAV)
TCA300 (Ad5)
TCA156 (Av3)
TLW045-016 (P4)
Clone 2
Clone 5
Clone 10
% hexon positive
Vp/mL
Normal Human Diploid Fibroblasts cells:
harvested 48 hours post-infection
TCA296 (OAV)
TCA300 (Ad5)
TCA156 (Av3)
TLW045-016 (P4)
Clone 2
% hexon positive
Vp/mL

Figure 7

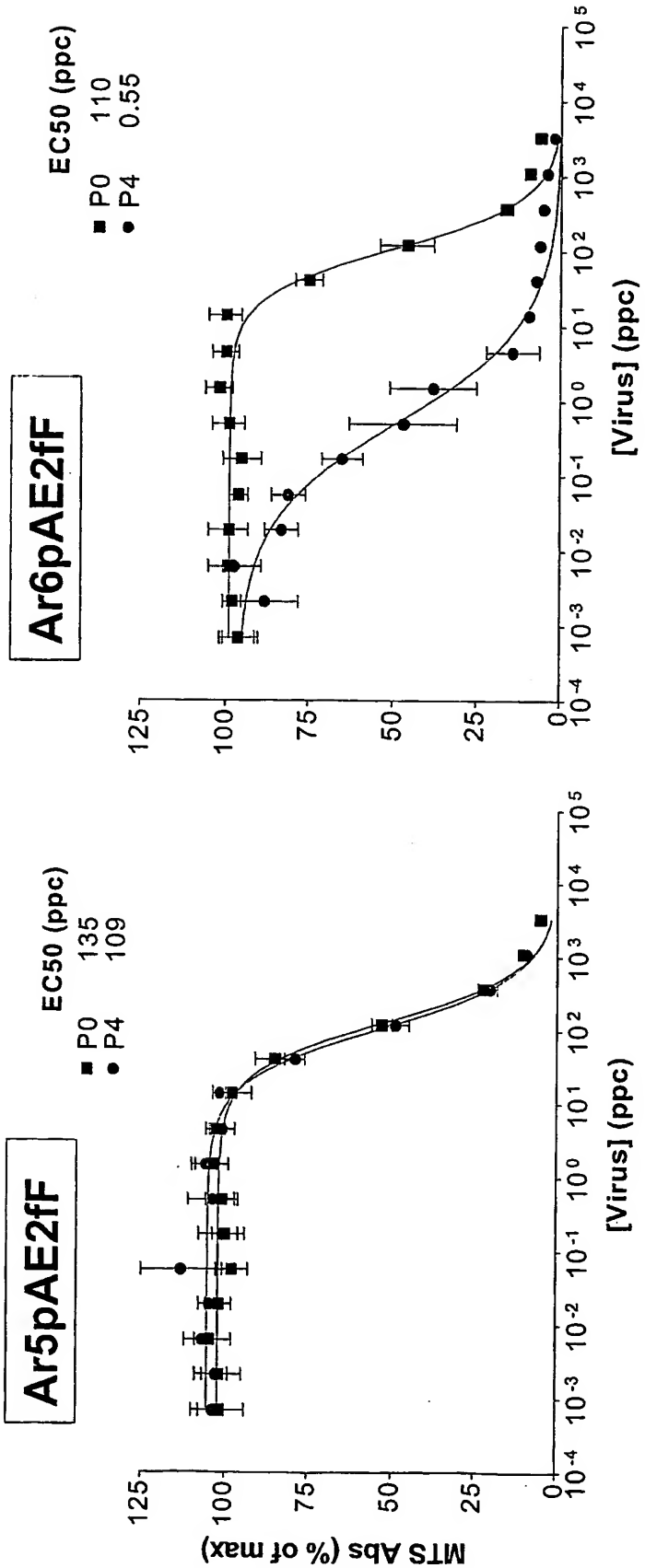
Ar5pAE2fF
EC50 (ppc)
P0 135
P4 109
Ar6pAE2fF
EC50 (ppc)
P0 110
P4 0.55
MTS Abs (% of max)
[Virus] (ppc)
[Virus] (ppc)

Figure 8

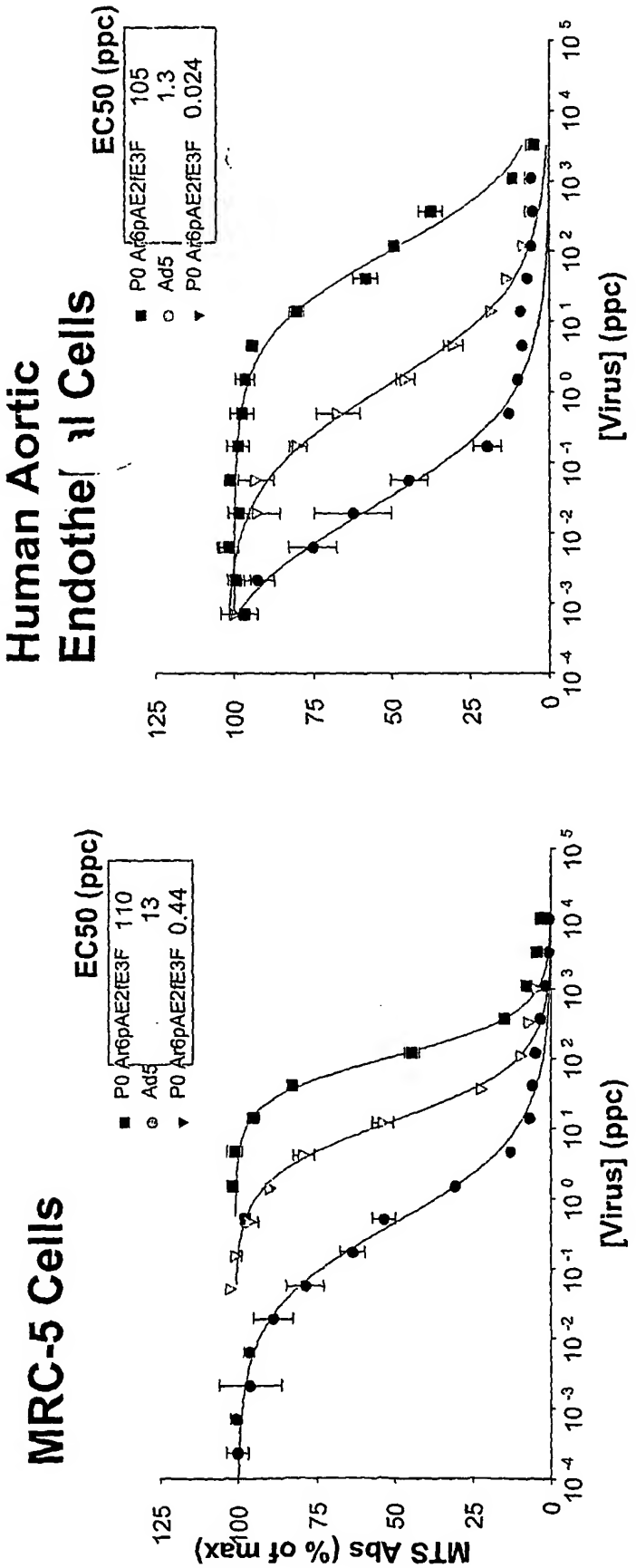
Human Aortic Endothel ıl Cells
EC50 (ppc)
P0 Ar6pAE2fE3F 105
Ad5 1.3
P0 Ar6pAE2fE3F 0.024
[Virus] (ppc)
MRC-5 Cells
EC50 (ppc)
P0 Ar6pAE2fE3F 110
Ad5 13
P0 Ar6pAE2fE3F 0.44
MTS Abs (% of max)
[Virus] (ppc)

Figure 9

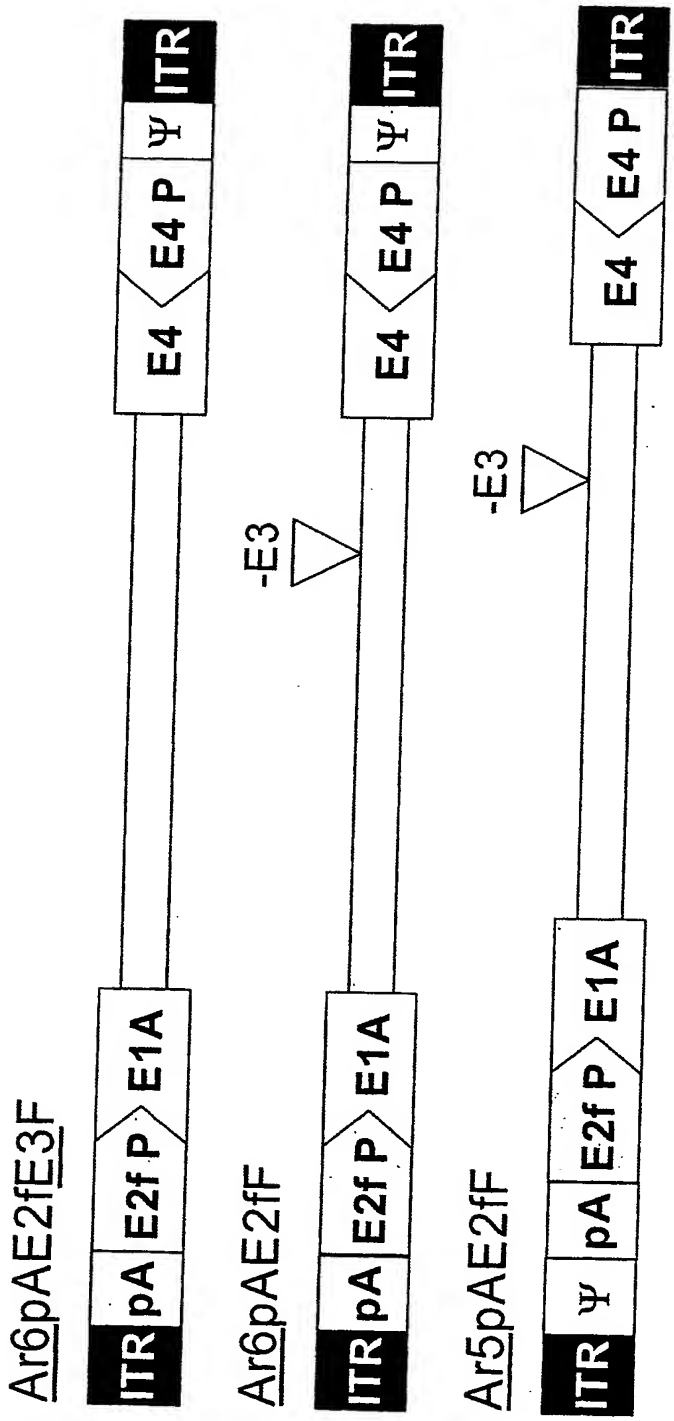
Ar6pAE2fE3F
ITR
pA
E2f P
E1A
E4
E4 P
Ψ
ITR
-E3
Ar6pAE2fF
ITR
pA
E2f P
E1A
E4
E4 P
Ψ
ITR
-E3
Ar5pAE2fF
ITR
Ψ
pA
E2f P
E1A
E4
E4 P
ITR

Figure 10

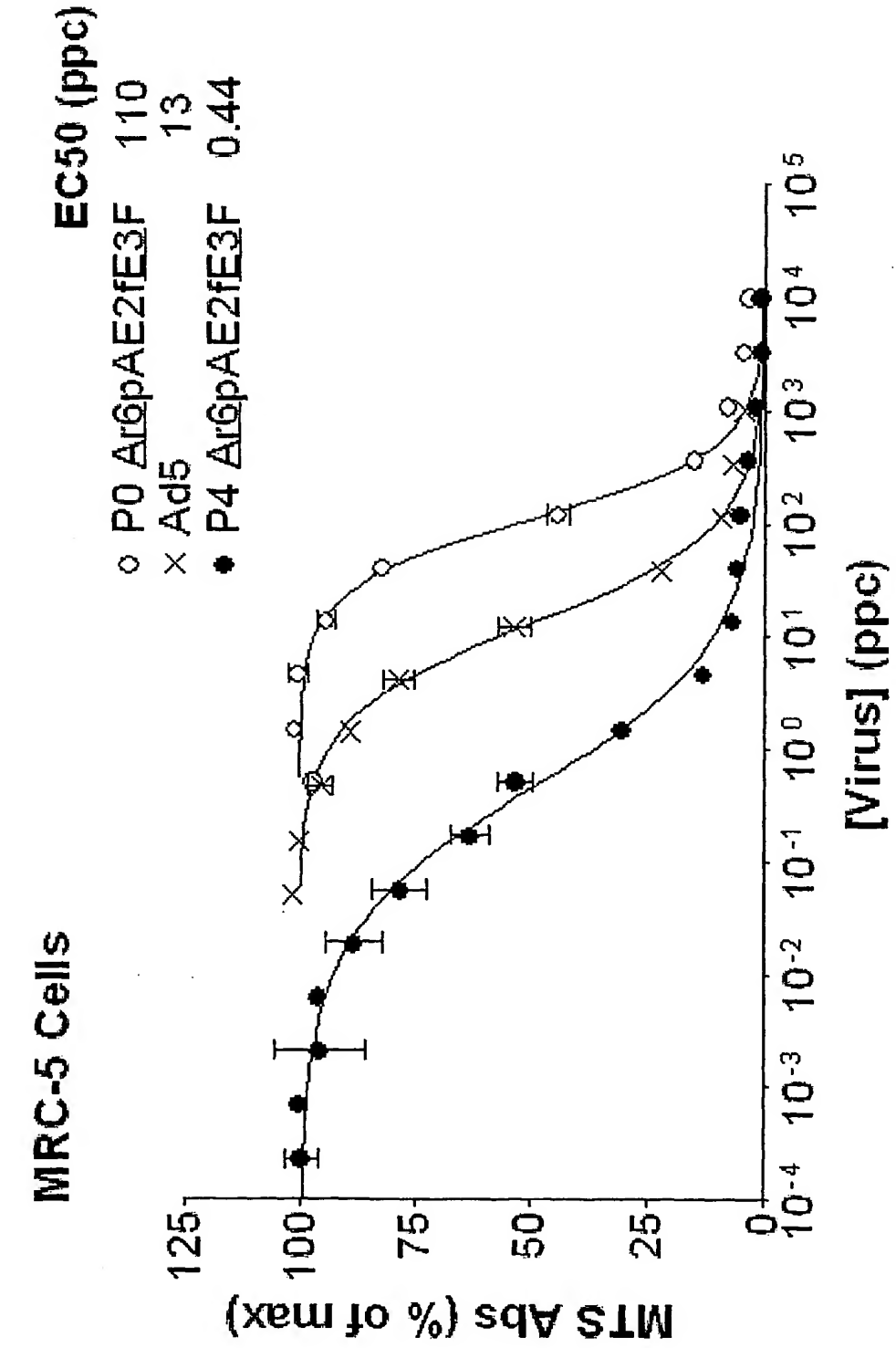
MRC-5 Cells
EC50 (ppc)
P0 Ar6pAE2fE3F 110
Ad5 13
P4 Ar6pAE2fE3F 0.44
MTS Abs (% of max)
125
100
75
50
25
0
[Virus] (ppc)
10-4
10-3
10-2
10-1
100
101
102
103
104
105

Figure 11

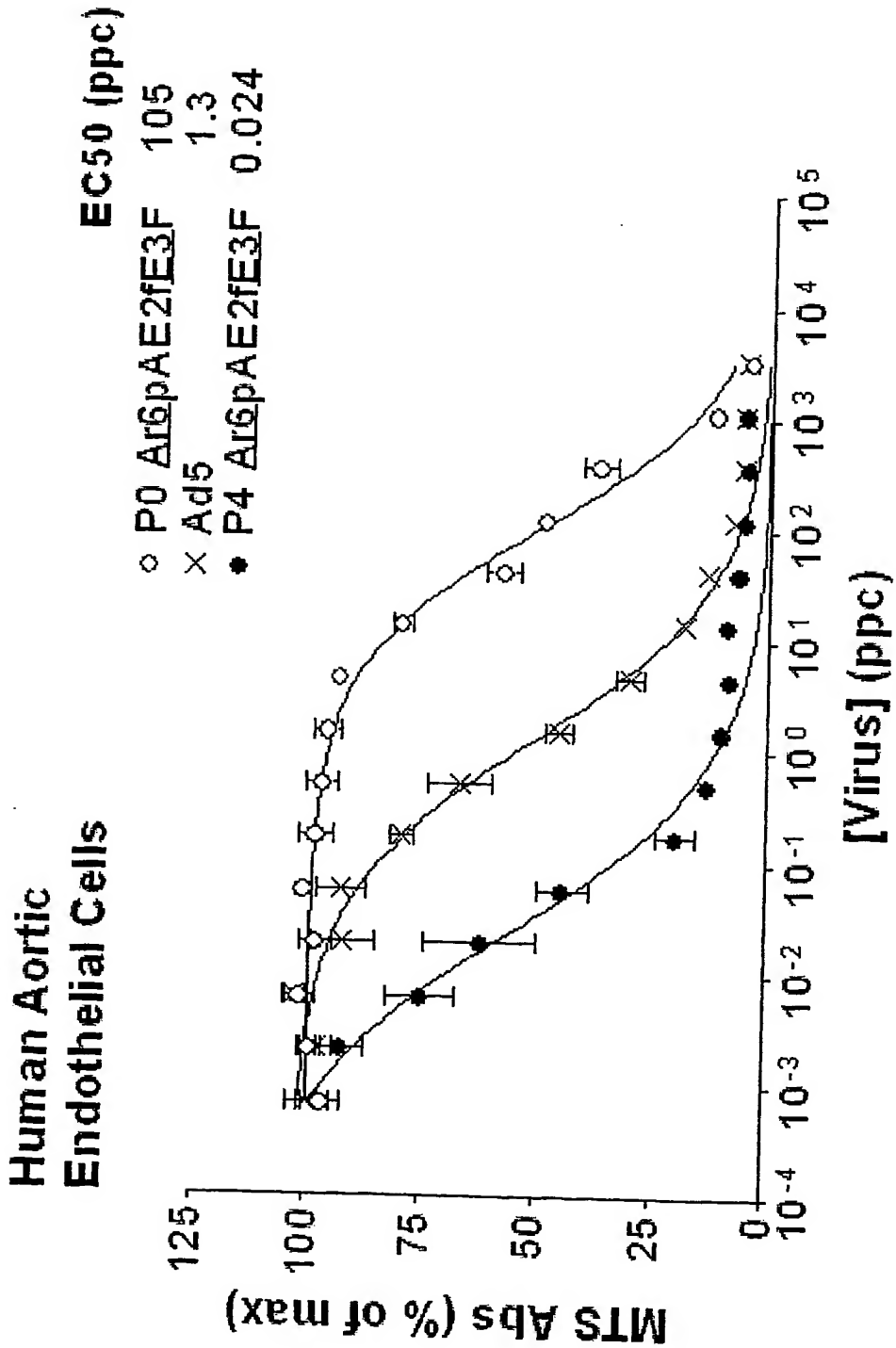
Human Aortic Endothelial Cells
EC50 (ppc)
P0 Ar6pAE2fE3F 105
Ad5 1.3
P4 Ar6pAE2fE3F 0.024
MTS Abs (% of max)
[Virus] (ppc)
125
100
75
50
25
0
10-4
10-3
10-2
10-1
100
101
102
103
104
105

Figure 12

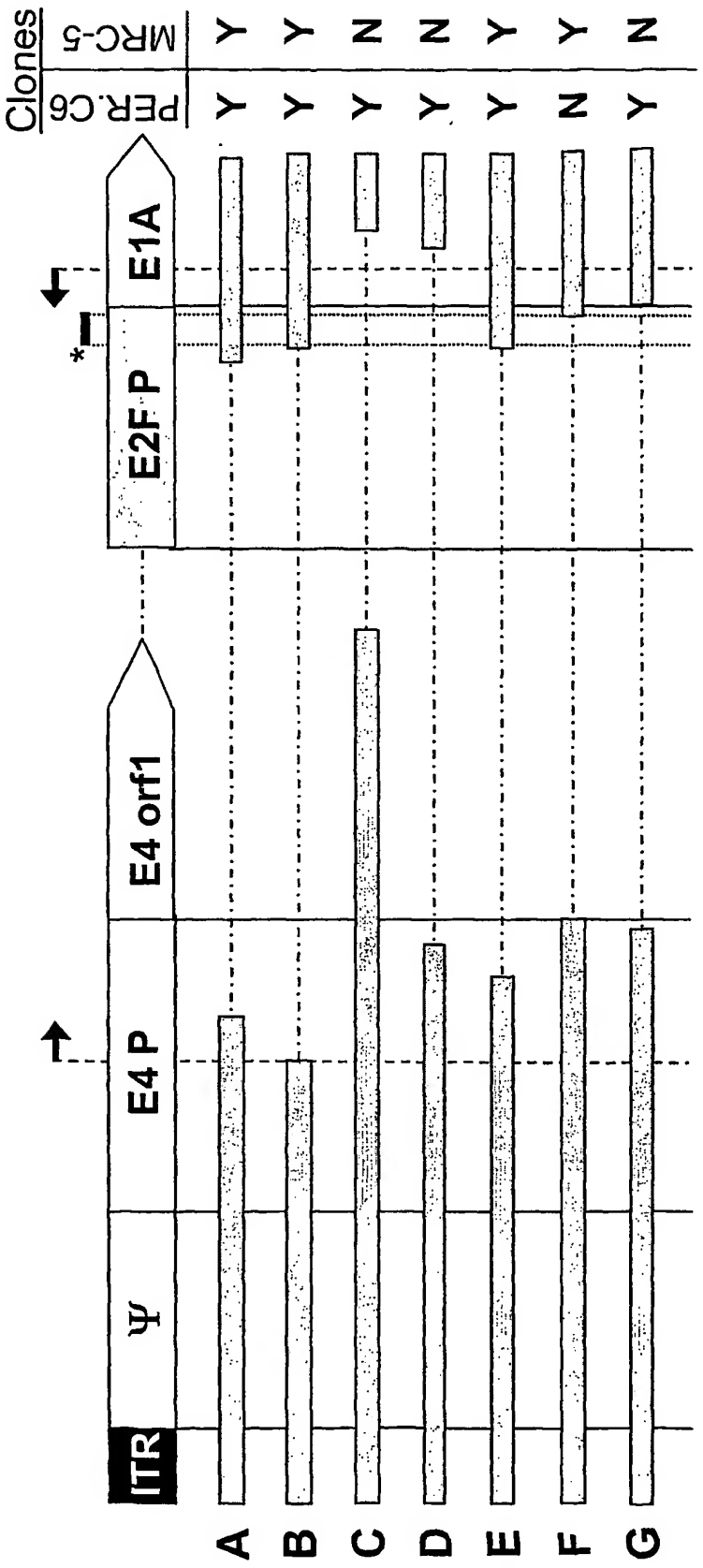
Clones
MRC-5
PER.C6
Y Y N N Y Y N
Y Y Y Y Y N Y
E1A
E2F P
E4 orf1
E4 P
Ψ
ITR
A B C D E F G

Figure 13

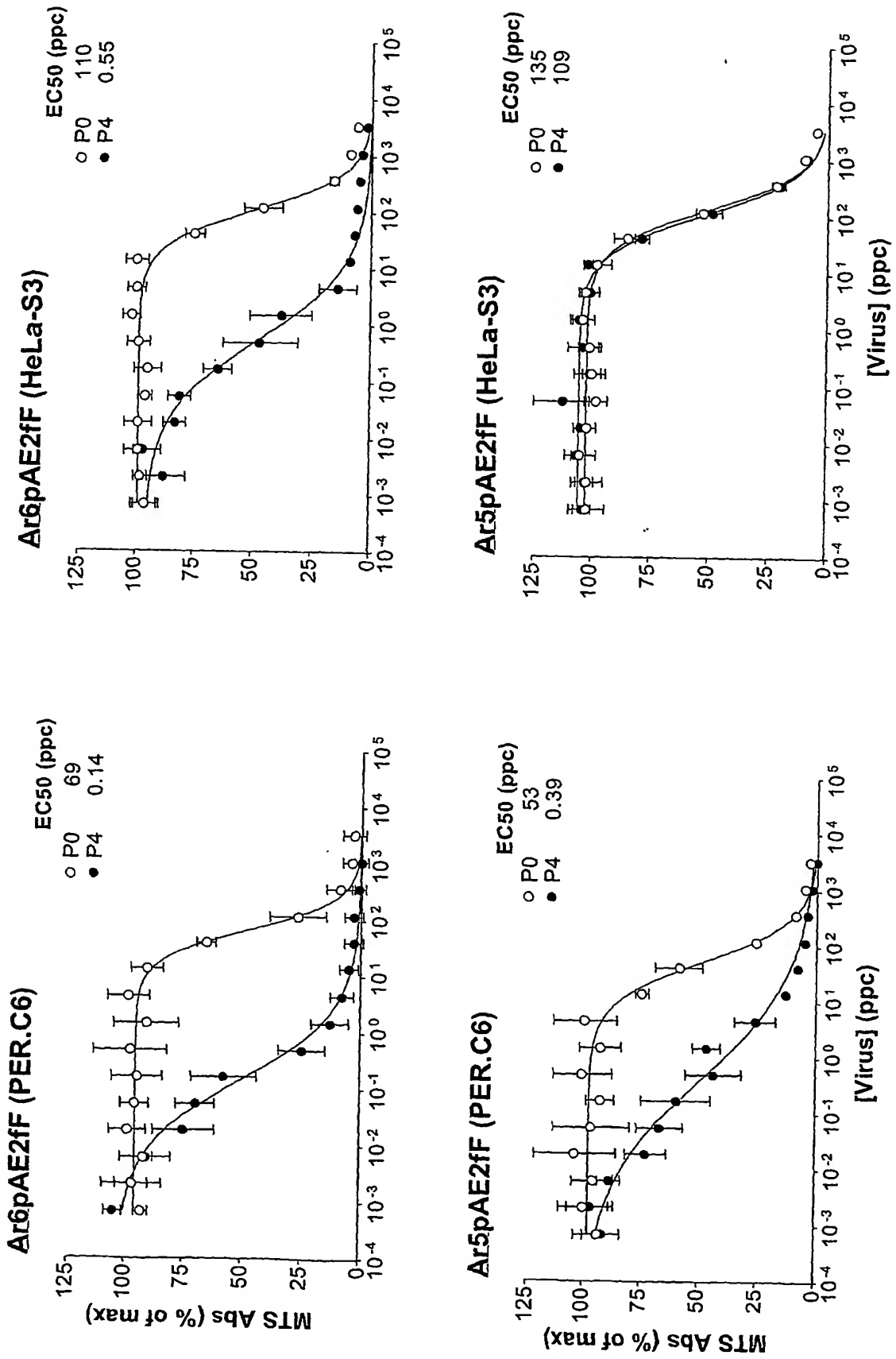
Ar6pAE2fF (PER.C6)
EC50 (ppc)
P0 69
P4 0.14
Ar6pAE2fF (HeLa-S3)
EC50 (ppc)
P0 110
P4 0.55
Ar5pAE2fF (PER.C6)
EC50 (ppc)
P0 53
P4 0.39
Ar5pAE2fF (HeLa-S3)
EC50 (ppc)
P0 135
P4 109
MTS Abs (% of max)
[Virus] (ppc)

Figure 14

SEQUENCE LISTING

<110> Bowe, Mark
Lyons, Russette
Walker, Tracey

<120> ASSAY TO DETECT REPLICATION COMPETENT VIRUSES

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<151> 2002-06-07

<150> US 60/459,375
<151> 2003-03-31

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<220>

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<212> DNA

<213> Artificial Sequence

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<211> 20

<212> DNA

<213> Artificial Sequence

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<210> 6

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<212> DNA

<213> Artificial Sequence

<220>

<223> Probe

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<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

<400> 7
tttctgggcg taggttcgc 19

<210> 8

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> PCR primer

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<210> 9

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Probe

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